

4 March 1982

Universities, profit and research

The presidents of five major research universities will meet privately later in the month to work out guidelines for commercializing research. They cannot be allowed the last word.

Dr Donald Kennedy, president of Stanford University, seems to have been granted his wish, frequently expressed a year ago, for a high-level conference on the problems of the relationships between universities and the commercial interests in recombinant DNA research. Later this month (and not far on the Californian coast from Asilomar), he and four other university presidents, (California, Caltech, Harvard and MIT) hope to assemble with select teams of their senior colleagues and industrial partners to hammer out guidelines for the proper conduct of joint commercial ventures. Although this occasion cannot match that in 1973 at which a relatively unstructured gathering of molecular biologists agreed, off their own bat, that there should be guidelines to regulate the conduct of recombinant DNA research, there is nothing reprehensible about what is now proposed. The five universities concerned are those which have been most intimately caught up with the commercialization of recombinant DNA research. What more reasonable than that they should arrange privately to meet to talk about their problems and to agree, if possible, to some common approach to the problems that have not yet arisen? There is no reason why they should be deflected from this sensible — and overdue — exchange of ideas by the wish of others, congressmen or other universities, to join the action.

Equally, however, it would be wrong for those planning to meet three weeks from now to think that what they agree among themselves about the proper relationship between universities and outside commercial interests will apply to other universities and other commercial interests. Indeed, the question is bound to arise whether any guidelines arrived at can be successfully applied even within the five participating universities — one of which, the University of California, is already in some difficulty with the state authorities on the customary exemption of academics who are also state employees from the standard constraints on conflicts of interest. This month's private meeting is bound to sharpen questions about the propriety of what the university intends. But there are also limits to the extent to which even the private universities can write their own tickets into a brave new-commercial future. Already, as the affairs of the University of California's Davis campus have shown (see p.6), the interests of graduate students and those who teach them are not identical — and, for a research university, the contentment of the graduate students takes precedence over that of the faculty. More generally but more importantly, the universities, especially the best among them, are public institutions embedded in a society which is perfectly entitled to views on how they should be run. And there are tax-exemption rules to give force to public opinion. This month's private conference could do a useful job, especially if it follows the sensible course of making public its conclusions. But it cannot be the last word.

What, in the circumstances, should Dr Kennedy and his colleagues talk about? Their first task must be to preserve the integrity of the institution of the university. Since the first excitement about the commercial potential of biotechnology five years or so ago, most universities have been in two minds about the roles that they should play. The prospect that they might, as a consequence of the cleverness of individual members of their faculties, be partly immunized from external financial pressures, has naturally been attractive. And government (not merely in the

United States) has been urging that universities should find other than public sources of financial support. But, dare it be said, not all biotechnology and not all biotechnologists automatically satisfy the criteria of excellence which universities, and especially the good universities, seek to apply to their affairs. How, then, to channel an institution's enthusiasm into support for some activity that will make a little money while giving proper academic attention to other activities that bring no profit? The ideal is that university faculties should be even more zealously encouraged than at present to decide among themselves between the good and the less good. The ideal is simple, but its practice will be hard.

Problems concerning people are likely to be even more important. It is essential that means should be found of making sure that universities cannot be fairly charged with misusing their students, perhaps by allowing faculty members to turn some piece of graduate research to commercial use. Similarly, because the effectiveness of universities requires a certain seamliness within the whole body of faculty and students, it is important that no single member of such a community should be thought capable of turning his academic position into unreasonable riches. And however intellectually absorbing may be the task of turning a bright idea into some commercial innovation, there must be a limit to the extent to which a university can let full-time members of its faculty function as entrepreneurs while remaining in the fold. Stanford has already made some headway with its internally promulgated rules for deciding how financial benefits from commercial enterprises should be shared between individuals, departments and the university. What is needed, now, is not so much a wider adoption of some standard formula as a better understanding of how such arrangements are working. Although biotechnology still seems the most conspicuous opportunity for turning academic work to profit, there are other better-established fields (engineering, for example) from which much can be learned. The trouble is that a clear understanding of this point will require an investigation, not just a two-day meeting.

British nuclear weapons

New nuclear submarines may be the best buy; talking to the French would be safer.

The British government's present dilemma about nuclear weapons policy is at least familiar. For at least the past twenty years, there has been continuing doubt about the most suitable means of preparing to deliver the modest British stock of nuclear explosives. It all seems so much more complicated than in the far-off 1950s when Britain, having become a nuclear power in a fit of absence of mind, was able to keep up with the superpowers with the help of home-built aircraft. Since then, it seems, there has been nothing but trouble. Knowing that a nuclear striking force is no more credible than the means by which explosives can be delivered to intended targets, the British government embarked on the development of intermediate-range missiles (remember *Bluestreak*?) and contour-following aircraft (the TSR2). But the pace turned out to be too demanding. This is why, in 1962, the British government leapt at the deal offered by President John F. Kennedy and took out an option first on the weapon called *Skybolt* (an early air-launched cruise missile that was promptly



cancelled) and then on *Polaris* missiles carried in British-built submarines. Now, with both the submarines and the rockets which they carry nearing the end of their useful life, the British government is doing what must now seem natural, and is planning to follow the United States in replacing *Polaris* by its more powerful (and accurate) successor, *Trident*.

The whole sorry tale should be an object lesson, and a warning, to other states flirting with the notion that they, too, might become second or third-rank nuclear powers. It should also help to moderate some of the more alarmist notions of how easily nuclear weapons might proliferate. For nobody believes that the cost of this still incomplete system will be contained within the £6,000 million (spread over fifteen years) that the Ministry of Defence is talking about. The argument that the total cost will be, in any case, only three per cent of the British defence budget over the fifteen-year construction period is hardly what matters — the cost works out at seven per cent of the likely equipment budget over that period. This prospect chimes ironically with the British government's decision to put several relatively new items of defence equipment on the secondhand market — the aircraft carrier *Invincible* is for example being sold at cost to Australia, while the Indian government is hoping to pick up an Antarctic research vessel (see *Nature* 25 February, p.640). Yet the cost of the British weapons programme, blessed by the Bermuda agreement, is undoubtedly far less than would be entailed by complete independence — as French taxpayers should be well aware.

The cost of being a serious nuclear power, even a small one, is onerous. The British government's domestic critics (who, on this issue, include a great many of its natural supporters and even one field-marshal) have made several telling points. Conventional defences will be weakened just when they need to be reinforced. The cost of *Trident* could better be spent on more creative projects. And now, for the first time since 1958, there is a risk that bi-partisan agreement that Britain should have some kind of nuclear defence force will disappear. No doubt each of these complaints carries some weight. The surprise, however, is that the argument between the British government and its critics has been confined to more or less tactical questions. Would cruise missiles be a better buy than *Trident*? (The answer is maybe.) Would the same expenditure on conventional forces contribute more to the defence of Western Europe? (Almost certainly yes.) The curious aspect of the row about *Trident*, however, is that so little is being done to clarify the reasons for and against the retention of an independent nuclear force of any kind.

In the 1950s, it was different. The decision then to develop an independent nuclear force stimulated a vigorous and informative argument. Serious opinion was split three ways. At one extreme were the unilateral disarmers, whose residual legatese seem now more anxious to prevent the siting of United States nuclear weapons in Britain than to re-examine British nuclear policy as a whole. Then, in the 1950s, what might be called liberal military opinion argued that British nuclear weapons might have a useful role in an escalating European conflict. The government view, which prevailed and has since stuck, was that an independent British nuclear force would help to cement the commitment of the United States to its European alliances, for it would be easier to stand aside from a conventional than from a nuclear conflict. Diplomatically, of course, the argument was never put so directly, for that would have invited the United States Congress to refuse to sanction the technical assistance with which the British nuclear force was built. Now, for what it is worth, the British case appears subtly to have changed. Last weekend, the Secretary of State for Defence, Mr John Nott, was arguing in a television interview that the *Trident* force, like and with its French equivalent, would come into its own if the pattern of alliances in Western Europe changed, leaving both France and Britain to look after their own defence.

This argument is essentially the same as the Gallois doctrine in the 1950s to justify the French nuclear force. To defend itself with nuclear weapons, a state merely has to equip itself with the means of inflicting damage on a potential enemy proportional to its own value as a prize. The argument is persuasive, but it conceals a serious danger — its universal applicability. If French and British

nuclear forces are justified in this way, why not Swiss or Swedish? The practical answer may be that at this late stage in the development of nuclear technology, the entry fee would be too high. But nobody can be sure. And if the British government is indeed edging round to planning for a world in which the pattern of European alliances may have radically changed, should it not now be talking to the French government, which must share the long-term interest that Europe should not become a collection of supposedly independent nuclear powers. And, by the same test, there is much to be said for going as slowly as possible with *Trident*, for the sake of all the money that may then be saved.

Chemical inspections

British proposals for verifying a chemical weapons treaty should be taken seriously.

The abiding trouble with arms control is that there is so much talk about it. Even if the apparently endless negotiations in Vienna on Mutually Balanced Force Reductions are forgotten (which is probably the most appropriate course), there are at present no fewer than four settings for serious discussions on the subject: the European Security Conference in Madrid (made necessary by the Helsinki agreements), the superpower talks in Geneva on European nuclear weapons, the standing Committee on Disarmament now also meeting in Geneva and the labyrinthine preparations for the United Nations session on disarmament, planned for June. Of these, however, only the two Geneva meetings are for the time being serious, and the United States and the Soviet Union have so far kept to their agreement not to broadcast news of what is going on. This is why it is refreshing that something sensible is happening at the other meeting in Geneva, that of the Committee on Disarmament.

The British government, often pilloried (as in the preceding account of its nuclear weapons policy), last week startled the Committee on Disarmament by tackling head-on one of the most difficult of the technical problems impeding the negotiation of a treaty to get rid of chemical weapons — that of verification. The problem is by now all too familiar. The agents potentially usable as chemical weapons can be made in manufacturing plants which are indistinguishable from those outside from the used for making pacific chemicals of various kinds. Although chemical munitions plants are by convention usually sited far away from civilian populations, there is no reason why their isolation should be ostentatious. The result is that remote surveillance, from satellites for example, cannot reliably indicate whether a state that has signed a treaty to dismantle its stocks of chemical weapons and to refrain from making more is actually keeping to the rules.

The British view, put forward last week, is that a sufficient degree of assurance against violations of a treaty could be provided by a judicious use of an expert committee, the monitoring of the flow of chemical raw materials through chemical processes, a complaints procedure and the judicious use of on-site inspection. Nobody can at this stage guess how the proposal will be received. The Soviet Union is notoriously sensitive about proposals for on-site inspection, but is not the only manufacturer of chemicals that is also touchy about its sovereignty. Sooner or later, however, on-site inspection is going to have to be a component of arms control agreements — and was, two years ago, accepted by all the nuclear powers to be a necessary part of the test-ban treaty then in draft. So the British government deserves encouragement for having raised the issue again. And it deserves some credit for trying to breathe life into an arms control project that has been too long forgotten since the superpower talks on the subject broke down in 1980. For this is eminently a field in which all potential parties to future conflicts would gain from an agreement not to manufacture weapons whose military value is as much in doubt as that of chemical weapons, whose production, maintenance and occasional disposal is a constant threat to the domestic population and whose agreed abolition would rid the world of a great nuisance and a needless cost.

Clouds on biotechnology horizon

Companies run short of sales and capital

Washington

The US biotechnology industry is coming down to earth with a bump, as various companies begin to experience difficulty in raising the capital needed to finance ambitious expansion plans.

Last week, for example, Bethesda Research Laboratories (BRL), one of the more successful of the many small biotechnology companies to have been set up within the past few years, announced that it was cutting back its workforce from 450 to just under 300 because of cash-flow problems, and was re-thinking plans for moving into larger facilities.

Only six months ago, the company was talking of a public offer of its stock, seeking to raise \$40 million to finance expansion over the next two or three years.

Other companies are experiencing the same problem, although none quite so dramatically. San Francisco-based Genentech, for example, is expected to have to think hard about how it is going to raise the new capital it will soon need for future expansion, although it said last week that at present the company was growing at its projected rate.

So far, out of more than 150 companies set up in the past few years, only one — Applied Genetics of Boston — is known to have folded. In most cases, according to Wall Street analysts, the cutbacks in growth being reported reflect a shake-out that had long been expected, partly a reaction against a period of over-expectation.

They point out that in the case of BRL, for example, the company is retaining most of its scientific personnel, particularly in the area of restriction enzymes for which it has established a worldwide reputation. The cuts are being made in more peripheral areas in order to maintain a healthy core.

The problems being experienced by the biotechnology companies are caused less by the current recession than by the fact that it is now much more difficult to raise new equity than it was even six months ago.

After the dramatic sale of stock in Genentech exactly a year ago, when shares rose from \$35 to \$80 on the first day of trading, shares have now stabilized around the original figure. Shares in Cetus, which went public with a far bigger proportion of its equity six months later, have slipped from \$25 to around \$13. The latest public newcomer, Collaborative Research of Waltham, Massachusetts, also received a relatively cool reaction when it went public last month.

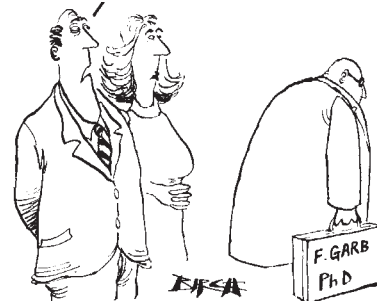
Investment analysts claim these figures reflect a new realism in the market, which recognizes that although genetic engineering techniques still offer a large commercial potential, there is still some way to go before anyone starts reporting a substantial profit. Two weeks ago, for example, Genentech reported that profits for 1981 were double those of 1980, but at \$300,000 these compare unfavourably with a revenue of \$21.3 million.

In the case of BRL, since the company is still private and therefore does not have access to large amounts of capital, it has had to rely more heavily on the sale of laboratory products, which last year generated an income of over \$10 million. This has made the company particularly sensitive to any fluctuations in the market, contributing directly to the decision to "restructure" its activities.

BRL is divided into four divisions covering research products, instrumentation, genetics research and molecular diagnostics. Staffing cuts are being distributed across all four divisions, and although ten PhDs were among those who had received their notice, the company says that in general the cuts had fallen less heavily on scientists (who make up about one-sixth of employees) than on other staff.

The cuts mark the end of a period of

"NEVER MIND—IF THINGS LOOK UP, WE'LL CLONE A FEW MORE."



spectacular growth for BRL. Formed by Stephen Turner, who previously worked with Becton-Dickinson, with two other employees in 1976 and based close to the National Institutes of Health which have been among its most important clients for restriction enzymes, the company had sales of \$350,000 in 1977, \$1 million in 1978 and \$5 million in 1980.

Asked in a newspaper interview last September how he viewed the future prospects for his company, Mr Turner admitted that the market for his products was rapidly filling up as the expansion of new genetic engineering laboratories levelled off. "I was taught long ago that the goal of business planning is survival, not dominance", he said. **David Dickson**

Cohen-Boyer patent extended

Washington

The US Patent Office last week informed Stanford University and the University of California, San Francisco (UCSF) that it has accepted their claims for a patent on any microorganism which is made using the genetic engineering techniques developed by Dr Stanley Cohen of Stanford and Dr Herbert Boyer of UCSF.

The techniques themselves were granted a patent just over a year ago. The patent was offered for licensing last summer, and last week Mr Andrew Barnes of Stanford's Office of Technology Licensing said 73 companies have so far agreed to pay the \$10,000 annual licence fee (five times which is credited against any future royalty payments).

Initially, applications for patents on both the techniques and the products of the techniques were filed simultaneously. However, in the light of the Patent Office's subsequent challenge to the legality of patenting living organisms — eventually resolved by the US Supreme Court, which agreed that they could be patented — the application was split into two parts.

Of the two, Mr Barnes said that the process patent already granted was considered by the universities to be the more significant. He added, however, that

the product patent, which it had been generally anticipated that the universities would receive, could strengthen their hand in any future legal proceedings.

For example, if the process patent alone had been allowed, foreign companies might have been tempted to import into the United States products made in countries where the techniques had not been patented, and the universities would have had to challenge this under a slightly ambiguous ruling from the International Trade Commission. Now that the product patent, expected to be formally granted in a few weeks, has been allowed, it will be a legal challenge to such imports.

The new patent will cover any recombinant plasmid made using the techniques developed by Dr Cohen and Dr Boyer, provided that the vector is compatible with the cellular host organism. The universities have also been allowed a patent on the transformant itself, providing this is a unicellular organism, such as a bacterium or a protozoan; human and plant cells, however, would not be covered.

Rights to the use of the products are already included in the licensing agreement which Stanford and UCSF have been negotiating with interested companies.

David Dickson

Pharmaceutical research

New tenants

The Swiss pharmaceutical company Sandoz has decided to set up a new research institute in the neurosciences at University College, part of the University of London. According to Mr E.J. Fullagar, director of Sandoz's UK operations, the new institute reflects the company's belief in the quality of British researchers — it hopes to recruit almost all its staff in Britain — and in the continuance of top quality British research. But the institute may help to persuade the British regulatory authorities to view more favourably applications for price increases on Sandoz's products.

The new institute, the first that Sandoz has set up on a university campus, is an experiment. According to Mr Fullagar, the venture will be no less expensive than building an institute on Sandoz's own premises. The hope is that spin-off from close links with the college will more than pay for any extra costs incurred.

The company chose to site its institute at University College partly because the two have collaborated successfully in the past on clinical studies and partly because the college's research in the neurosciences matches its own interests. The new institute will be chiefly concerned with the role of neurotransmitters and neuromodulators in the nervous system.

Although the institute will be entirely independent of the college, employing

about 60 of its own research staff, the plan is that there should be a close relationship between the research staffs and that the college might look to some institute staff for teaching.

The college, which although relatively well endowed is nevertheless suffering from university cutbacks, will benefit financially. Although Sandoz's rent is only nominal, the company will pay the college a £2.5 million premium and thereafter pay for overheads and services such as library and catering facilities. The money will help the college out of some of its difficulties, in particular those of completing capital projects abandoned because of cuts in its grants from the University Grants Committee. Thus work begun on a new chemistry building in the early 1970s will now be completed. Sandoz will refurbish the building vacated when the chemistry department moves into the new building to be used by the new institute.

Sandoz estimates that it will spend £5 million on setting up the institute, divided equally between payments to the college, the costs of refurbishing and the cost of equipping laboratories. The laboratories will not be ready before September 1983 at the earliest. Hence, according to Mr Fullagar, it is still too early to describe the research programme in detail, or to say whether the college and institute will cooperate on joint programmes and, if so, how. Patents taken out on the results of research supported entirely by the institute will, however, belong to Sandoz. But where research has been jointly supported, the college's lawyers will advise.

Sandoz, therefore, seems cheerful about the long-term prospects of British university research. Its view, however, is not shared by Biogen, the Swiss-based pharmaceutical company, which has chosen not to site its third laboratory in Britain on the grounds that the university cuts may irreparably damage British biotechnological research.

Biogen plans to set up a new laboratory along the lines of those at Cambridge, Massachusetts, and Geneva. The idea is that the laboratory should be separate from but near to a university department with considerable expertise in recombinant DNA research. The company says that it cannot justify setting up a laboratory near a suitable British university because no department is guaranteed to escape the ill effects of the university cuts. So it is looking elsewhere, in particular in Belgium and West Germany.

The new laboratory is intended to complement the work of Biogen's existing laboratories and to allow the company to spread its research between institutions employing no more than 75 research staff. According to Walter Gilbert, Biogen's chief executive, Biogen will nevertheless consider employing British biotechnologists of suitable calibre who apply for posts in any of its laboratories.

Judy Redfearn

Pesticide data

Row on access

Washington

The latest move in the lengthy battle over access to scientific data on the medical effects of pesticides came last month with the US Environmental Protection Agency (EPA) asking Congress for authority to write its own rules for the release of such information. Members of the House of Representatives' Agriculture Committee have tried to find a compromise between public interest groups demanding open access to the data submitted to the agency and pesticide manufacturers, who argue that the unrestricted release of such information would benefit their commercial competitors.

The manufacturers have been pushing for changes in the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA), first passed by Congress in 1972, which provides any individual with access under the Freedom of Information Act to scientific and medical data submitted to EPA as part of the regulatory process of registering a new chemical. Attempts at revision are being fought by a coalition of health groups, conservationists and labour unions which argues that the demands being made by industry would make it impossible, for example, for research scientists to communicate openly the results of their analysis of the data.

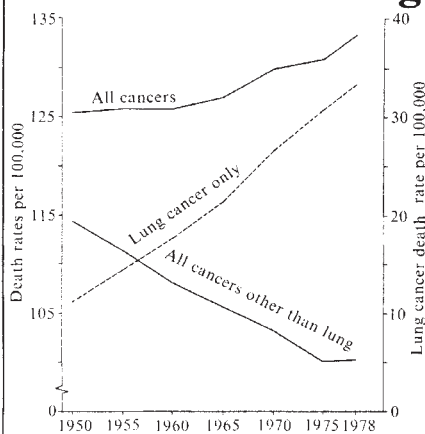
In its zeal to respond to public protests about the potential health hazards of new pesticides, Congress passed in 1978 stiff new amendments to FIFRA. These included a specific clause saying that data on the medical effects of a pesticide were not to be treated as a trade secret, and were not therefore exempt from freedom-of-information requests.

Since then, manufacturers working primarily through their Washington-based lobbying organization, the National Agriculture Chemicals Association (NACA), have tried to challenge the clause several times in court. The companies claim that the data produced in their research laboratories are private, and that the government's requirement that they be handed out on demand is a violation of constitutional rights.

So far, the courts have upheld the validity of the new law. The manufacturers have responded by turning their attention first to Congress, in the hope of having the law revised, and more recently to EPA with the prospect of receiving a more sympathetic hearing.

Last summer Mr George Brown, previously a prominent member of the House Science and Technology Committee and now chairman of the research sub-committee of the House Agriculture Committee, introduced a new bill that would limit the conditions under which the data could be released. These revisions would make the data available only to approved

Cancer and smoking



As shown in the figure, published in "The Health Consequences of Smoking: Cancer 1982" (US Department of Health and Human Services), during the period 1950 to 1978, the overall cancer death rate increased. This trend results from a remarkable increase in lung cancer death rates. However, if lung cancer deaths are excluded, the overall cancer mortality rate has fallen. This is largely a result of improved diagnosis, treatment and survival times for cancer sites not associated with cigarette smoking, such as prostatic, colorectal and breast cancers, while lung cancer survival remains dismal.

individuals, who would have to take notes from reports made available in EPA reading rooms. Labour union representatives and people working in the health services would be able to borrow complete reports, but would not be allowed to divulge the contents to others. No employee of another chemical manufacturing company would be allowed access to the data; and nobody would be allowed to make a complete transcript of the research reports.

Members of the subcommittee staff argue that such provisions, provide a compromise between industry's desire to protect confidential research data, and the public's right to information on the risks and benefits of chemicals used for crop protection. Both parties in the dispute disagree.

The manufacturers were initially enthusiastic about the changes proposed by Mr Brown, but their support has evaporated as the amendments have been revised to take into account some of the objections of those claiming to be defending the public interest.

Environmentalists and labour union groups are seizing this "window of opportunity" between the failure of the last industry challenge and the eventual tightening up of administrative requirements to get as much information on existing pesticides out of EPA as they can. EPA administrator Anne Gorsuch informed a coalition of such groups that the agency intended to respond to demands for pre-registration data on eleven chemicals as well as data on alternatives to 2,4,5-T.

At the same time, EPA has been talking with industry representatives, who have asked for an "administrative stay" on the release of data under the Freedom of Information Act, and are said to be helping EPA to develop a set of restrictions that would be both legally defensible and commercially acceptable. **David Dickson**

Moves in Mongolia

The new president of the Mongolian Academy of Sciences is Dr Choydogiyn Tseren, a 42-year-old physicist. Dr Tseren, who is a graduate of Moscow University, has spent a considerable time (1966 to 1971 and 1974 to 1980) at the International Nuclear Research Institute at Dubna. In 1980, he returned to Mongolia to become academic secretary of the academy.

Dr Tseren's appointment as president of the academy, which was effected by a special decree of the presidium of the People's Great Khural (parliament) of Mongolia, followed the dismissal, at the beginning of the year, of the previous president, Dr B. Shirendev, for his "misunderstanding" of the nature of socialism, a term which apparently covered hard currency irregularities.

Vera Rich

Coal gasification

Belgian first

Brussels

A gleam of hope for Europe's coal production came with the announcement this month that the first gasification of coal at depths of nearly 1,000 metres would take place this May in Belgium. The technique has been used for some time in the Soviet Union and in other countries but never at these depths. If the project at Thulin near the French border lives up to its promise it will turn many of the inaccessible or abandoned coal deposits in Europe into a new source of energy.

The Thulin project is run by the Institute for the Development of Subterranean Gasification, 60 per cent of whose funds are provided by the Belgian and German governments, the other 40 per cent by the EEC.

The retrocombustion technique used works by burning the coal *in situ* and then pushing the gas through the coal strata to a second outlet. The creation of such a circuit at this depth means working with high pressures and ensuring that the coal is permeable enough to allow the passage of the gas to the second bore hole. Air is forced down the first bore hole and ignited at a temperature of some 400°C, so that the coal ignites spontaneously.

The problem is that the deeper the coal lies the further away the second bore hole must be. At 1,000 metres, the gas would have to travel between 80 and 100 metres. The costs rise with the depth of coal deposits, as it becomes increasingly difficult to complete the circuit. Oil can be extracted at depths as low as 4,000 metres, but with coal the deeper one goes the more impermeable it is. At Thulin the problem has been overcome by forcing compressed air along the vein in order to enlarge the existing natural fissures.

The commercial applications of the technique are still uncertain, as it depends not only on energy prices but also on the coal seam thickness. Nevertheless, the British Coal Board is following the experiments with interest as a potential means of exploiting North Sea coal.

So far the gasified coal being brought to the surface in Belgium is of low quality, yielding some 2,500–3,000 kcal per m³. However, specialized treatment could increase this to 12,500 kcal per m³. At first, therefore, the gasified coal would simply be burnt in power stations but there is speculation that it could one day compete with pressurized liquid gas — now used to run cars.

The earliest date by which the technique could bring commercial benefits is put at 1990. Nonetheless the Belgian energy minister, Etienne Knoop, seizing on a spark of optimism to brighten his country's current troubles, remarked, "We are today inaugurating the birth of a new natural source of riches for our country".

Jasper Becker

Dutch energy policy

Talking shop

Waalre, The Netherlands

The steering group set up in The Netherlands to referee the national debate on the country's energy problems now finds itself grappling with financial constraints — and with some political disenchantment.

The long-running and often acrimonious Dutch energy debate began in the early seventies with the government's "energy memorandum", but got under way in earnest only last June, with the appointment of a nine-strong steering group of politicians, energy experts, scientists and labour union representatives, given the brief of organizing the public discussion.

Some years ago, a budget of £3.5 million (Dfl. 17 million) was allotted to the steering group to cover total costs for the two-year discussion, and the group now has a staff of 14 academics (including a theologian) and 17 administrators. Most of the money was intended for subsidies to the various groups in society seeking to work out alternative energy policies to that put forward by the government.

In January this year the steering group awarded a first round of grants, totalling £1.5 million, to 31 groups including a consortium of 50 environment groups and employers' organizations. This was only half the amount asked for by the applicants, and reflects the financial pressures being felt by the steering group. In February the government decided on a budget of around £5.5 million for the steering group instead of the £7.4 million requested, of which £1.8 million is intended for subsidies, not the £3.1 million asked for.

Discussions on the budget revealed political discontent over the policies of the steering group, discontent which at one time threatened to bring an early end to the project. Some members of parliament and press commentators were critical of the way in which the first batch of subsidies was allocated — in particular, complaining that too much money (roughly half the total) went to pro-nuclear groups having close ties with industry, and in no desperate need of the cash.

The "information phase" of the discussion is now due to be completed by the end of 1982. The aim of this part of the process is, in the words of steering-group chairman Maurits De Brauw, a former minister for science policy, "to give the public the unique chance to present alternative, scientifically based policy ideas, equivalent to those of the establishment". When that is over, a further short public discussion will follow, probably concentrating on three or four energy/economy scenarios selected from the initial phase.

Whether or not this attempt at involving all segments of Dutch society in a major

decision will actually yield some clear results remains to be seen. But parliament has at least decided to await the final report from the steering group, expected at the end of 1983, before making its final decision.

Casper Schuurin

Commercialization of research

Student dilemma

Washington

Renewed public controversy has arisen about the connections of various faculty scientists at the University of California's Davis campus with a local research company Calgene, particularly over the effect these could have on graduate students.

Last October, plant biologist Dr Ray Valentine was told by the university that he must either relinquish his position as chief investigator on a \$2.3 million grant to the university's experimental station from the Allied Chemical Corporation to support his work on nitrogen fixing (*nif*) genes, or give up his contracts with Calgene, of which he is a co-founder and vice-president (*Nature* 8 October 1981, p.417).

The university's ultimatum followed the discovery that Allied Chemical had also agreed to buy a 20 per cent equity interest in Calgene. Dr Valentine subsequently agreed to step down from the university research project. However, the publicity helped spur the state's Fair Political Practices Commission into recommending that university researchers should no longer be allowed a blanket exemption from conflict-of-interest laws routinely applied to other state employees.

The latest dispute has arisen over charges that some graduate students in Dr Valentine's laboratory have been placed in a difficult position since it became clear that some of their research on the genetics of nitrogen fixation could have significant commercial consequences.

The campus daily student newspaper last week published extracts from memoranda written by faculty members expressing concern about the impact of Dr Valentine's involvement with Calgene on the academic functioning of the university.

One set of memoranda relate to a decision by four of the five graduate students to transfer their research, with Dr Valentine's approval, to another laboratory on the campus. Dr JaRue Manning, professor of bacteriology, said that graduate students have revealed several common concerns. The first was Dr Valentine's statement that it was the students' responsibility "to meet with Calgene personnel and establish that their graduate research project is not being conducted at Calgene".

The second factor quoted by Dr Manning was what he described as the switching of student research projects as a condition for continued support after students have spent up to two and a half

years on a project. "Regarding the second factor, while there is certainly a need to coordinate research activities with objectives stated in grants, etc., the manner in which these changes were made raise a number of questions as to the actions taken."

Other memoranda raise more general issues about the effects of intensifying links between university researchers and companies such as Calgene. In a note to Dr Charles E. Hess, dean of the College of Agricultural and Environmental Sciences, Dr Emanuel Epstein, professor of plant nutrition at Davis, suggests that scientists are no longer keen to exchange research data with each other as soon as it comes off the scintillation counter or the electrophoresis cell.

In another memorandum to Allen G. Marr, dean of graduate studies and research, professor of zoology Robert L. Rudd complains that plans by Calgene to initiate a research project which competes with the dissertation research objectives of one of his graduate students was "an infringement on academic freedom and potentially harmful to graduate education in general".

Both Dr Epstein and Dr Rudd said last week that they were not opposed, in principle, to collaboration between university research scientists and the private sector. As a land-grant college, the Davis campus of the University of California has long had close links with both local farmers and agricultural companies. Both, however, were concerned that public reaction to the tensions caused by the recent rush to exploit research findings in genetic engineering could lead to bureaucratic controls that might themselves be an infringement on academic freedom.

The state's Fair Political Practices Commission met on Monday to discuss whether it should give final approval to the suggestion, approved by a 3 to 2 majority at its January meeting, to require university researchers to make public any financial interest they may have in a company which is sponsoring their research (*Nature* 4 February 1982, p.357).

The internal memoranda appear to have been leaked as part of a campaign by the California Rural Legal Assistance to persuade the commission to go further and require university scientists to disclose all their financial commitments in outside companies, whether or not the companies are providing research funds to them.

Dr Valentine did not return telephone calls last week asking him to comment on the questions that had been raised about the position of the graduate students. A spokesman for Calgene said that that the company did not employ any Davis students, and was not sponsoring any research at the university. (About thirty Davis faculty members are reported to be acting as consultants to the company.)

David Dickson

Polish crisis

Questions asked

One of the casualties of martial law in Poland has been the free flow of information between Polish scientists and their Western colleagues. With the total severance of telecommunications, and the postal service considerably delayed by censorship, it has been difficult, if not impossible, for scholarly contacts to be maintained. Moreover, reports in the Western media of protest strikes in the universities and institutes of the Academy of Sciences have aroused considerable anxiety among scientists abroad as to what exactly is happening to their Polish colleagues.

The main source of information has been the lists of internees appearing from time to time via official or unofficial channels. The first list — issued by the Military Council for National Salvation — was clearly erroneous; it included several names of people who were actually outside Poland when martial law was declared, such as the young chemist, Miroslaw Chojcecki, who became active in the underground press after his dismissal from the Swierk nuclear research institute in 1977. Later lists, received through church channels, though fuller, are still far from complete, and evidence of a colleague's being at liberty is, by and large, negative, and deduced from the absence of his or her name on the lists to date.

Only in a few cases, like that of the 101 intellectuals who signed a letter to the Sejm (parliament) calling for an end to military rule, or the published list of academics called in for consultations with General Jaruzelski in December, is there positive evidence.

A number of scientists have been approaching Polish embassies and consulates to enquire after their colleagues. In the case of known activists, such as the mathematical logician Dr Janusz Onyszkiewicz, a presidium member of the Mazowsze (Warsaw area) branch of Solidarity, or the economist Dr Bronislaw Geremek, one of Solidarity's chief "advisers", such enquiries were initially met by suggestions that if a job could be found for them abroad, the Polish authorities would not refuse an exit visa. Following General Jaruzelski's assurance in the Sejm on 25 January that no internee would be deported from Poland, such proposals have been dropped. Nevertheless, enquiries at consulates do not proceed smoothly — officials are unwilling to discuss "rumours" — even to deny them.

On the facing page we print some extracts from such a conversation, lasting several hours, between Dr Wilfred Hodges and Dr Derrick of the British Logic Colloquium and the Polish Chargé d'Affaires Mr Gorajewski and Scientific Counsellor Mr S. Wojtaszek at the Polish embassy in London.

Vera Rich

Diplomatic conversations

What follows is an extract from a record by Dr Wilfred Hodges, of the British Logic Colloquium, and officials of the Polish Embassy in London.

Question: In his statement of 13 December 1981, General Jaruzelski said: "Our nation has enough strength and wisdom to deploy an efficient, democratic system of socialist rule. In such a system military forces will be able to stay at their proper place — in the barracks." On the other hand we have heard that some university buildings in Poland are now being used as barracks. . .

Answer: Where have you heard that university buildings are being used as barracks? This is sheer rumour and we do not answer rumours.

Question: In the same statement, General Jaruzelski said: "The full list of those interned will be published." When?

Answer: Sixty-three names were published soon after 13 December, and further names have been given since on Warsaw Radio. In any case, out of the 5,906 people interned, 839 have already been released.

Question: It is reported that Solidarity members are being required to sign a "loyalty pledge" dissociating themselves from Solidarity. Can you please tell us the wording of this pledge, and what the penalty will be for those who refuse to sign?

Answer: In Poland some state administrators have always been required to sign a statement of loyalty. This is nothing new, and we have heard nothing about any change in the text of the statement. As for other people being asked to sign it, and what may happen to them if they refuse, we have no sources of information apart from the British press. We have never heard mention of anybody being asked to sign a pledge which mentioned Solidarity.

Question: Will all academics who are released from internment without facing criminal charges be allowed to go back to the jobs which they held before 13 December 1981?

Answer: We don't know, because we don't know about the loyalty pledge.

Question: We have heard that the following logicians have been interned: Konrad Bieliński (Warsaw) in Biatoteka; Janusz Onyszkiewicz (Warsaw) in Strzeblinek; Marian Sebrny (Warsaw) in Biatoteka; Jan Waszkiewicz (Wrocław); Andrzej Wroński (Kraków). Can you confirm this information?

Answer: We have no list of internees. We notice that a high proportion of the people you mention were in KOR (Workers' Defence Committee). Do you have any contacts in Poland other than these people? «Answer: Yes, many.»

Question: Are the academics who are interned being allowed visits by their families? Do they have writing materials

and the use of scientific books and papers?

Answer: Family visits to internees are allowed. The internment camps are resort centres, so there are reasonable facilities, but we would hardly suppose the circumstances are conducive to research. The internees get exercise and see the official newspapers. We would assume that an internee can get books and papers from his home by asking a relative to collect them.

Question: Will you allow a British logician to visit the logicians who are interned, so that he can let us know the conditions in which they are kept, and so that he can pass on to us any requests from them?

Answer: All visas issued before 13 December 1981 were automatically cancelled when martial law was imposed. The present regulations are that if anybody applies to us for a visa, he has to state which organization in Poland is supporting his visit, and then we forward the application to Warsaw for this information to be checked. A British logician could certainly apply to us, but unless he could say that, for example, the Polish Academy of Sciences was inviting him, we suppose that his request would be refused.

Question: If British logicians give you letters (either in English or in Polish) addressed to logicians interned in Poland, will you guarantee that these letters will be delivered, and that the internees' replies will be delivered to us?

Answer: Mail is allowed through now, both within Poland and between Poland and the West. But all mail between Poland and the West is subject to censorship. At a time of crisis, the bureaucrats who operate the censorship are not going to make fine distinctions; if anything doubtful comes past them, they are just going to say no. But we expect that things will get gradually better. If you want to get in touch with internees, try writing to their families.

Question: New regulations for the Polish universities have recently been announced. Are these new regulations permanent, or do they automatically end when the present emergency ends?

Answer: New regulations for the universities were under discussion before 13 December and a new bill was before parliament, but martial law prevented it from being enacted. Since it was necessary to reopen the universities somehow, we reverted to the regulations based on the 1952 law. But this is a temporary situation, and new laws on the universities will be passed as soon as possible.

Question: Which items of academic and scholarly work are to be censored? Will lectures in the universities, and letters to

colleagues abroad, be censored?

Answer: Academic censorship is now the same as it was in the 1960s.

Question: After the present emergency, will scholars who are invited to visit universities or attend conferences abroad be allowed to travel outside Poland, or will permission be granted only to those who have the correct political attitude?

Answer: Even in the present emergency, some Polish scholars are leaving Poland to visit abroad. If a particular employer in Poland asks for permission for one of his employees to travel to the West, he can certainly apply, and in principle he might get permission. But visas issued before 13 December were automatically cancelled at the imposition of martial law. If there are one or two Polish scholars that you would like to have visit you, please do apply. For example, the Polish Cultural Institute in London would very likely be willing to bring over one or two Polish scholars for a meeting to be held at the institute.

Question: In 1980 the authorities in Prague cancelled a major international conference of the Association for Symbolic Logic five weeks before it was due to take place, and no clear reason has yet been given for the cancellation. Already one philosophical exchange between Oxford University and the Polish Academy of Sciences in Warsaw has been postponed by the British Academy as a result of the military rule in Poland. In this climate, are the Polish authorities aware what harm would be done to East-West scientific cooperation if the International Congress of Mathematicians, due to meet in Warsaw in August, had to be cancelled or moved as a result of the situation in Poland?

Answer: We shall try to get facts about any conferences you ask us about. In particular we shall ask whether the Polish authorities are still planning for the following conferences to go ahead: Conference in Functional Analysis, Stefan Banach Institute in Warsaw, February 1982; International Congress of Mathematicians, Warsaw, August 1982.

● Mr Gorajewski and Mr Wojtaszek made a number of other points, including the following: (1) The Polish authorities had no option but to impose martial law, in order to protect Poland's economic links with the other countries in Eastern Europe. (2) By writing to the British Foreign Office to complain about the internal affairs of Poland, the British Logic Colloquium has violated the Helsinki Accords.

● On receiving a copy of the above summarized answers, Mr Wojtaszek wrote: "Since many of the answers have been significantly misquoted and/or taken out of context, it would at least be fair to say that you, and you alone, might accept responsibility for the interpretations you made and which are often in sharp contrast with the contents of the answers you received."

CORRESPONDENCE

Back to Nature

SIR — The article "Mental stress given environmental status" (*Nature* 21 January, p.179) shows an extreme ignorance of the dangers of ionizing radiation. Stating that there was "no significant extra radiation exposure to individuals" after the Three Mile Island incident implies the existence of insignificant levels of radiation. Not true! Any amount of radiation will produce a proportionate increase in mutation rates in exposed genes. There is no safe level of radiation, only less harmful levels.

Your leading article (*Nature* 21 January, p.177) regarding the recent US Court of Appeals ruling that psychological effects must be considered when making an environmental impact statement is speckled with specious statements and biased comments. First, it is ridiculous to presume that under this ruling "hydroelectric schemes of all kinds would quickly be stopped". Nuclear projects are fundamentally more dangerous than hydroelectric facilities. Dams do not increase mutation rates; dams do not remain mutagenic for thousands of years. Second, you lament that "most defence projects and all nuclear projects (would be quickly be stopped)". These structures should exist to serve the majority of the people of this country (not the armament industry or the utilities), and if the people feel traumatized by these projects to the extent of working to prevent their construction, then the people should have the power to say No. You feel that this result "would be a nonsense", and that "environmentalists (now) have a licence to do irreparable damage". I fail to see how allowing people to shape the nature of their environment can be "nonsense" or irreparably damaging to anything but the armament and utility industries. Finally, your choice of adjectives suggests an empathy with industry, not the environment. Metropolitan Edison is not "luckless". Luck has nothing to do with the scientifically and economically unsound decision to build nuclear power plants. Please, consider diverting your sympathy from the nuclear industry to your namesake: Nature.

CHRIS Q. DOE

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Astronomical flaw

SIR — Can someone explain why creationists seem to reserve their attention for biologists and the theory of evolution and ignore astronomers?

As I understand it, the creationists reject the theory of evolution because that is not how things are described in the Book of Genesis. Yet that book clearly states that the Earth, complete with flowering plants, was created before the Sun (*Genesis* 1, v.9–19).

I am no astronomer but I should have thought that that was incompatible with any currently accepted theories of planet formation.

However, I have not seen the point raised in any creationist argument.

Are some parts of *Genesis* more historically true than others? If so how does one tell which is which?

I.P. FREEMAN

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Beijing duck now

SIR — As reported in four articles in *Nature* of 3 December 1981¹, in 1980 foreign scientists were allowed entry into Tibet for the first time since the Chinese military takeover of that country in 1950. This opening of Tibet for scientific study is highly welcome. However, the Chinese usage of special Chinese spellings of Tibetan words (as also used in the *Nature* articles) merits comment.

Tibetan is a language quite unrelated to Chinese and has, in contrast to the latter, an alphabet. This can be very accurately transcribed using roman characters. However, Tibetan spelling being fairly complex, it is often preferred to spell Tibetan names the way they sound, so that an English-speaking reader can immediately pronounce the names with a fair degree of accuracy.

Unfortunately the Chinese now have extended the pinyin system also to Tibetan words. This system was created to romanize Chinese words in a unified manner, and it is the official system in use in the People's Republic of China today. (Thus Peking is now spelt Beijing.)

Since pinyin was constructed for the Chinese language, it can be used for Tibetan words only by a concomitant loss of accuracy. This is partly because of the system's inherent adaptation to the Chinese language, and partly because pinyin spelling is the result of a foreigner (Chinese) writing in roman letters in an artificial system what he thinks Tibetan words sound like. This filtering necessarily introduces a considerable degree of distortion.

Furthermore, pinyin replaces well-known spellings with strange ones. For instance, the second largest city of Tibet, traditionally spelt Sigatse, becomes Xigaze. Since "Shigatse" very accurately reflects the Tibetan pronunciation and is the established way of spelling the name, nothing is gained by the substitution. The same goes for changes of other words into pinyin.

Finally the Chinese system introduces a further confusion in that other Tibetan-speaking areas, such as northern India and Nepal, Bhutan, Sikkim and Ladakh, do not use this system. Thus the danger ensues that two parallel romanizations will be used for the same words.

It is to be hoped that intensified research contacts regarding Tibet can be established in the future between the West and China, and ultimately also Tibetan scientists. The question of the spelling of Tibetan words would then become of increased importance.

JAN ANDERSSON

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1. *Nature* 294, 403; 405; 410; 414 (1981).

Missed the mark?

SIR — Jon Marks' letter (*Nature* 28 January, p.276) shows a misunderstanding of the intent and meaning of the Bible. The Bible is a collection of writings from various peoples and times, all focusing upon their experience of God. It is not designed to be a scientific textbook. It is wrapped within the time, culture and world outlook of its authors (what literature is not?) and yet succeeds in breaking out of those limitations to accomplish its purpose and point men to God's active concern in their lives. In this respect, Dr

Marks is just as guilty as those he criticizes in quoting the Bible in a way that is incompatible with its primary intent. He does it for ridicule; his opponents for scientific reductionism; but is either legitimate?

The way in which biblical quotations are used often tells us more about the person quoting than the subject under discussion. This is clearly evidenced by the way in which *Leviticus* 11:19 and *Luke* 23:43 were utilized. In his reference to the bat being categorized as a bird, Dr Marks has not discerned the culturally bound attempts of the writer of *Leviticus* to make sense out of the world based upon the evidence as he understood it. We should not stand in judgement of such attempts simply because of our more privileged information. One wonders if some of our scientific "certainties" will pass the scrutiny of scientists in four thousand years' time. Again, an understanding of the relationship between Jesus' death and the Kingdom of God in the world would prevent misinterpretation of *Luke* 23:43. "Paradise" as an image of the Kingdom of God is inseparably linked within the New Testament of Jesus' death and resurrection. That crucial phase of Jesus' life was beginning to take place on that very day ("today"). If the creationists and evolutionists would take time actively to study the Bible then such matters would not come to trial.

The Bible does see the design and diversity of nature as pointing to a Creator, but does not demand a fundamentalist, creationist interpretation of *Genesis* 1. Even Creationism should allow an element of evolution. Nature suggests that the Creator builds one design from another to achieve increasing complexity.

Furthermore, the Bible clearly indicates that God is still creating. The Thomistic argument of design pointing to the Creator is admittedly not without its difficulties — but equally so is the argument from chaos to non-creator. Both fall under the same criticisms and fail to see that either is dependent upon faith in establishing the relationship between data and that to which data point. Evolutionary theory has for too long been six feet above contradiction. In this context the challenges enshrined in neo-Lamarckism, punctuated equilibria theory and cladistics should be welcomed. Evolution should be subjected to the same scrutiny as any other theory, and should be capable of modification or replacement. Indeed when a theory loses its ability to be superseded it becomes a dogma.

The evolutionary process, when extrapolated beyond its scientific confines, can be used to justify any number of monstrous evils, as history only too well documents. We must admit that man is a creature whose life is not encapsulated within empirical study alone but contains a drive which pushes him Godwards. Man can only be understood as making progress in so far as the touch of God is able to continue to direct him. The way forward to us seems to be commitment to scientific investigation combined with dedicated and honest study of the Bible.

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NEWS AND VIEWS

Processes of gene duplication

from Alec J. Jeffreys and Stephen Harris

GENE DUPLICATION is probably one of the most important mechanisms for generating new genes and novel biochemical functions during evolution. Evidence for this process is seen in the numerous families of related proteins specified by the genomes of higher organisms. Through the use of recombinant DNA methods to study the arrangement of duplicated genes, two basic patterns of gene family organization have emerged: linked clusters of related genes in which individual genes are separated by long regions of non-coding DNA, and dispersed families in which individual members are scattered over widely separated chromosome locations.

The globin family in mammals neatly illustrates both modes of organization, with two unlinked gene clusters, one specifying the α -related globins and the other the β -globins. The family has recently been the subject of intensive research which has attempted to discern the molecular mechanisms that lead to the tandem duplication of a single gene and to the dispersal of genes to remote locations.

The human β -globin gene cluster contains five active globin genes arranged in the order ϵ - γ - δ - β , together with two pseudogenes and extensive tracts of DNA between the genes¹. The two closely related fetal genes (γ and δ) have clearly arisen by a recent duplication within the cluster. Recently, Smithies and colleagues have sequenced over 11 kilobases (kb) of DNA encompassing the fetal globin genes, and have shown that the region contains a large (5 kb) tandem duplication. Each duplicate has a γ -globin gene embedded within it^{2,4}.

How did this duplication of a relatively large chromosome segment arise? A clue comes from the existence of a short directly repeated DNA segment (r) at each end of the repeat, giving an organization r- γ -r- δ -r (ref.3). While the authors suggest several models to account for this arrangement, the simplest involves mispairing and unequal crossing-over at meiosis between the repeated elements present in an ancestral r- γ -r DNA segment

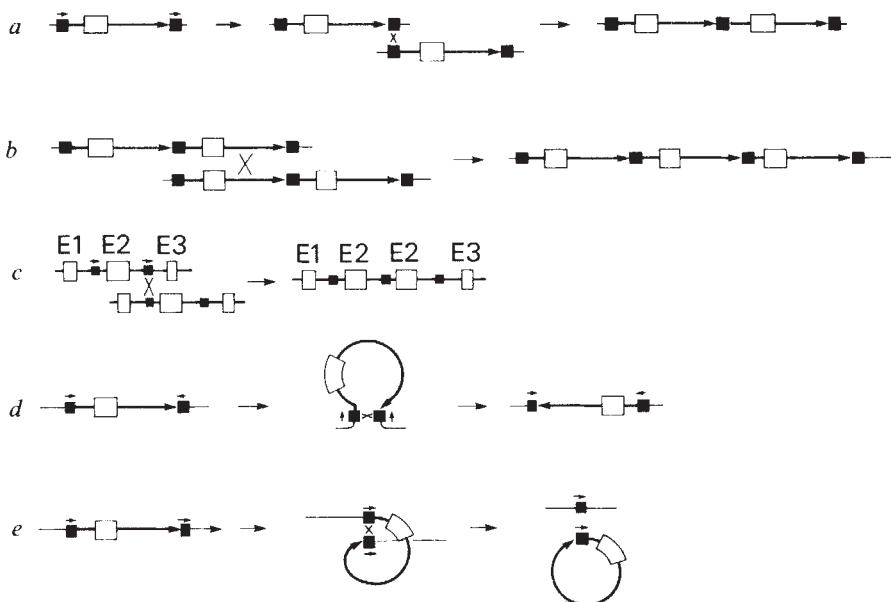
(Fig.1a). If this is correct, then the size of the duplicated region would have been initially determined by the (fortuitous?) spacing of repetitive elements near the non-duplicated locus. The prevalence of families of short repetitive elements scattered throughout the mammalian genome would suggest that large block duplications might occur commonly, though the proportion of these that would be fixed in evolution is not known.

According to the locations of the repetitive elements involved in unequal crossing-over, more than one gene could be included within the duplicated block. A fascinating example has been found by Cleary and colleagues in the β -globin gene cluster of the goat⁵. The adult Λ -globin gene is preceded by a pseudogene $\psi^Z\beta$, and similarly the ϵ -globin gene coding pre-adult globin is linked to another closely homologous pseudogene, $\psi^X\beta$. The authors suggest that this arrangement has probably evolved by two successive duplications of a single ancestral β -globin gene. The first duplication, accompanied

by silencing of one duplicate, gave a $\psi\beta$ - β pair, and more recently a larger block duplication of this pair generated the $\psi^Z\beta$ - $\Lambda\beta$ and $\psi^X\beta$ - $\epsilon\beta$ pairs. The length of the block duplication and the presence of repetitive elements flanking the blocks have not yet been determined.

By comparing duplicated DNA segments it is possible to learn something of the modes by which genic and intergenic DNA diverge in evolution. The γ - δ -globin gene duplication provides an excellent example. Phylogenetic comparisons of primate β -globin gene clusters show that the duplication probably occurred about 20–40 million years ago in the lineage leading to Old World monkeys, apes and man⁶. Since then, the sequences of most of the two block repeats have diverged about 14 per cent from each other and have also accumulated numerous very small deletions and insertions^{2,4}. Surprisingly, one substantial region (1,500 base pairs long) including most of the γ globin gene is more than 99 per cent identical in sequence to the corresponding

Fig.1 Consequences of recombination between dispersed repeated DNA elements. Short repeated DNA sequences (solid boxes) are shown either near genes (open boxes in a, b, d and e) or within intervening sequences separating the exons (c) of one gene. In d, the short sequences are orientated in opposite directions.



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region around the γ -globin gene. Since it is difficult to see how selection could have led to this remarkable homology, the authors suggest that the region has been homogenized by gene conversion between misaligned γ -globin repeats very recently in evolution^{2,3}. Gene conversion between related DNA sequences seems common in evolution and appears to operate not only within protein-coding gene families but also in middle and highly repetitive DNA sequences. It may serve to maintain sequence homogeneity within a family (see ref. 7). It is interesting that the end of the converted region in the γ -locus, as well as the end of a long homology block around the duplicated α -globin gene in man, contains the simple sequence (TG)_n^{2,8}. Since this sequence can, in principle, adopt a left-handed helical configuration, the authors suggest that such an unusual localized structure in DNA might be involved in initiating the related processes of recombination and gene conversion.

In the goat, the duplicated loci each containing a $\psi\beta$ - β -globin gene pair provide an interesting test for sequence divergence of homologous pseudogenes. When homologous functional genes are compared with each other, base substitutions are found preferentially in flanking sequences, introns and third codon positions in exons, presumably because substitutions that alter the amino acid sequence of the protein are more likely to be eliminated by selection. In contrast, the goat $\psi\beta$ and $\psi\gamma$ sequences show throughout a much more uniform divergence⁵, consistent with the idea that pseudogenes are the non-functional relics of excessive gene duplication (see ref. 9). However, it is worth noting that what is a pseudogene in one species can be a functional gene in another; the minor adult δ -globin gene is active in man, apes and New World monkeys but has been silenced independently in Old World monkeys¹⁰ and lemurs¹¹.

The presence of small repetitive elements and tandem repeats in gene clusters can lead to other shifts in gene organization. Long tandem repeats provide excellent targets for unequal crossing-over, and can rapidly amplify to give arrays of three, four or many more closely related genes (Fig. 1b), such as those found in ribosomal DNA clusters, histone gene clusters and satellite DNAs. Oppositely orientated short repeats can cause localized inversions (Fig. 1d) and may have been responsible for one form of thalassaemia in man¹². Dispersed repetitive elements can also exist in introns, and recombination between direct repeats in different introns (Fig. 1c) could lead to an internally duplicated intron/exon arrangement that could be further amplified by the mechanism in Fig. 1b. Strong evidence for internal duplication has been found in IgC_H¹³, ovalbumin¹⁴, ovomucoid¹⁵ and α -fetoprotein¹⁶ genes, and further amplification is probably responsible for

the very highly split nature of the α -collagen gene¹⁷.

Dispersed repeated elements could also lead to excision of genes via intrachromosomal recombination (Fig. 1e). The excised circle could re-integrate elsewhere in the genome by homologous recombination with another member of the repeated DNA family. While this process might contribute to the dispersal of gene clusters without duplication, other mechanisms involving gene duplication also seem to operate. The simplest processes entail chromosome duplication, polyploidization and chromosomal translocation. A more subtle and remarkable process that might both duplicate and scatter genes is being unravelled (see A.J. Flavell *Nature, News & Views* 295, 370; 1982; and ref. 18). A mouse α -globin pseudogene with two remarkable properties has been found: first, it has escaped the parent gene cluster; and second, it appears to have been processed to remove introns, as though the gene had passed through an RNA splicing stage. The discovery of retrovirus-like sequences near this processed $\psi\alpha$ -3-globin gene in the mouse genome suggests that the original gene was somehow kidnapped by a provirus and transposed via spliced retroviral RNA.

Clearly, gene clusters are inherently unstable entities that can expand, contract and disperse during the course of evolution. Of all the possible shifts in arrangement, natural selection will eliminate the unfavourable, leaving neutral and advantageous changes to be fixed in evolution. Although it is difficult to distinguish between neutral and selective changes, some degree of adaptive changes has accompanied the γ - γ -globin gene duplication in man and the β - β duplication in the goat, in which the duplicated genes tend to be expressed at different stages of development. The molecular basis for these adaptive changes is as yet completely unknown, and is unlikely to become clear from DNA sequencing data alone. □

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Parasite-induced pathology

from F.E.G. Cox

INFECTIONS with parasites are characteristically long and chronic and the resulting diseases involve diverse pathological mechanisms. The damage caused is vastly in excess of the expected local reactions to the parasites themselves and appears out of all proportion to their size. A recent colloquium* attempted to assess some of the mechanisms involved.

For many years it has been believed that the prolonged but misdirected immune responses of the host are largely responsible for the pathological changes observed. In some infections this is no doubt the case; in schistosomiasis, for example, the adult worm, coated with host antigens, is immunologically invisible but the eggs lodged in various tissues elicit classical delayed-type hypersensitivity reactions which result in the formation of granulomas, the deposition of collagen and eventually fibrosis that is often fatal. This is not, however, the whole story.

Schistosome egg antigens are themselves able to stimulate the production of collagen in a manner similar to that seen in many diseases — in particular silicosis, in which silicon compounds stimulate macrophages to produce factors that activate fibroblasts to produce collagen and eventually fibrosis. The collagen in schistosome granulomas is a normal host component produced in the liver from arginine which is used for the synthesis of proline, an essential prerequisite for collagen formation. Normally, proline is degraded but this degradation is inhibited within schistosome-induced granulomas. Collagenolytic activity is restored after the infection has been cured. Thus the major pathological lesion in schistosomiasis can be partially explained in terms of parasite by-products that influence the normal physiological activities of the host. The egg antigens also seem to be directly hepatotoxic and the granulomas actually protect the liver cells from damage, which suggests that this pathological reaction, induced by either or both egg antigens or immunological reactions to egg antigens, may have some protective effect not yet clearly understood. The clearance of foreign immune complexes is also inhibited

*An international colloquium on the pathogenesis of disease induced by parasites was held at the Prince Leopold Institute of Tropical Medicine, Antwerp, on 11-13 December 1981. The proceedings, *From Parasitic Infection to Parasitic Disease*, will be published by S. Karger (Basel) in the Series *Contributions to Microbiology and Immunology*.

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in schistosome-infected animals; this is due not simply to changes in circulation resulting from the granuloma formation, but to the presence of adult worms. The parasites themselves are thus responsible to a large extent for the pathology associated with schistosomiasis.

In fascioliasis it is now clear that excretory products from the adult flukes have a direct deleterious effect on the liver cells of the host by blocking mitochondrial ATP synthesis. In African trypanosomiasis, the trypanosomes catabolize aromatic amino acids and not only deplete the host of essential amino acids but also produce toxic by-products from them. The main catabolites of tryptophan, for example, bring about major physiological changes including the induction of a state of coma similar to that seen in human sleeping sickness and those of phenylalanine inhibit gluconeogenesis and mitochondrial activity. In animal trypanosomiasis, extravascular parasites cause cellular infiltration and necrosis, particularly in the heart and nervous system.

Cardiac and nervous tissue are also involved in the pathology of malaria, and in rodent models, endomyocardial fibrosis following the passage of macrophages, polymorphs and fibroblasts through the endothelial layer has been demonstrated after drug cure and some brain lesions appear to be caused by the compression of capillaries by the accumulation of fluid in pericapillary glial cells. These observations indicate that physiological changes may contribute to the pathology of malaria but other evidence suggests that immune complexes characteristic of malaria simply indicate brain damage and do not necessarily cause it.

The overall conclusion is that we now know enough about the pathology of parasitic infections to say that physical and physiological changes may be as important as, or even more important than, immunologically induced damage. Of particular importance is the realization that certain aspects of the pathology of sleeping sickness resemble phenylketonuria, of schistosomiasis silicosis and of malaria, paraquat poisoning or burns. Our understanding of the pathology of parasitic infections can benefit from progress being made in other fields and should not be considered in isolation. Again, certain techniques devised for parasitological studies such as the injection of Sephadex beads loaded with schistosome egg antigens to induce liver granulomas may be relevant to the investigation of non-parasitological diseases. In this context, it is important that parasitological studies have had a leading role in our understanding of the part played by eosinophils in various infections. As eosinophils are involved in endomyocardial fibrosis it may well be that the role of these cells, and the basic proteins they produce, will become more clearly

defined as a result of further investigations using parasites.

Eosinophils are not the only cells to be incriminated in the pathology of parasitic infections — macrophages and their products seem to play a central part in many infections. We need to know much more about the activities of these cells before we can say that we understand the basis of the pathology of parasitic infections let alone know enough to ameliorate the effects. Nevertheless, there is no doubt that pathology is about to emerge from years of comparative neglect when it has taken second place to immunology and will become one of the most important and rapidly developing aspects of parasitology. □

A new approach to the determination of absolute configuration

from W. Jones

CHIRALITY plays an important part in chemistry and lies at the heart of many important biological processes. But under what circumstances is one kind of handedness chosen? Is a particular molecular glove intended for the right or the left hand? And even more fundamental, from which side of the mirror do we observe? These questions have been asked ever since Pasteur's classical experiment in 1848 resolved the two optical isomers of sodium ammonium tartrate¹.

Notwithstanding Pasteur's breakthrough the question of absolute configuration still remained, it being necessary to make an arbitrary assignment (either right or left) to one particular molecule and refer all others to this. No further progress was made until 1949 when Bijvoet proposed the use of anomalous dispersion of X rays². His proposal rested on the fact that for an appropriate wavelength of probing X-ray beam a non-centrosymmetric crystal structure will lead to slight differences in scattering intensities from certain planes, in violation of Friedel's law. Experimental proof followed and since then the absolute configuration of over 500 optically active compounds has been determined by this method³. Apart from two other techniques³, one rather esoteric and the other somewhat restricted, but which incidentally confirm assignments based on Bijvoet's proposal, no other general methods have been available by which absolute handedness may be

determined. In this issue of *Nature* (p.21 & p.27) however, Leiserowitz and his colleagues describe a new approach whereby absolute configuration can be deduced from a study of the crystal habit which a collection of the chiral molecules adopt. The approach has its origins in the reactivity of organic molecules within crystalline solids.

The course of many solid-state reactions is crucially controlled by the arrangement of the molecules within the crystal lattice⁴⁻⁶. Furthermore, any molecular asymmetry is manifested in the solid state by the manner in which the molecules pack in the highly ordered three-dimensional arrangement of the crystal. If both types of optical isomer are taken up within a crystal then symmetry operations which 'translate' a right-handed molecule into its left-handed counterpart (that is, a mirror plane) must be present. If such symmetry features are absent then only molecules of one handedness may be incorporated and the space group (that which describes the three-dimensional arrangement) is said to be chiral. Furthermore — and herein lies its strength — for any solid-state reaction which proceeds under so-called lattice control this chirality will be transferred to the product and only one type of optically active isomer will be formed.

By this method absolute asymmetric syntheses have been accomplished⁷, with the chirality of the product determined by the absolute configuration of the parent crystal. The next stage, however, is to enquire whether there exists a feedback mechanism whereby 'seeding' of the mother liquor with product of one (say D) chirality will induce preferential crystallization such that even more of the D isomer may be formed. The results indicate that the addition of D product invariably leads to growth whereby L crystals (and subsequently L product) are in excess — the inversion rule.

Leiserowitz *et al.* show that this occurs because the D-product isomer is preferentially absorbed on growing D-reactant crystals and impedes or considerably modifies their growth. In the absence of optically active impurities, perfect crystals of D and L form are obtained from solution. After addition of a small amount of D impurity only L crystals with perfect morphology emerge. But the full implication of these observations lies in the possibility of determining absolute configuration. For if one uses 'impurity' molecules with identified groupings and observes which crystal face or faces are modified with respect to particular directions within the crystal one has a method of obtaining absolute configuration. This argument (see p.27) leads to the emergence of a new and potentially powerful technique capable of dealing with racemic crystals and which relies only on observation of crystal morphology and very elementary X-ray diffraction measurements. The prospects are exciting.

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Not only may we find here a convenient method of ascertaining absolute configuration, but we may also reap other practical benefit from the ability, which their work presents, of engineering the morphology of crystals. □

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3. See, for example, Dunitz, J. D. *X-ray Analysis and the Structure of Molecules* (Cornell University Press, 1979).
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C-reactive protein and the acute phase response

from M. B. Pepys

THE acute phase response consists of the increased production of a number of plasma proteins which occurs following most forms of tissue injury, inflammation, infection and malignant neoplasia. C-reactive protein (CRP), so-called because it precipitates pneumococcal somatic C polysaccharide, was the first 'acute phase protein' to be identified. It was discovered in O.T. Avery's laboratory at the hospital of the Rockefeller Institute and was one of three fundamental contributions to biology to emanate from there, the others being the identification of DNA as the genetic material in pneumococci and the establishment of the role of polysaccharides as antigens.

There has lately been much progress in the characterization of CRP, of other acute phase proteins and the mechanisms underlying the acute phase response in general. These advances were marked recently by the first international meeting* to be held on the subject.

Many circulating proteinase inhibitors, complement, coagulation and transport proteins have been shown to behave as acute phase reactants. The general nature of the acute phase response, its stimulation by many forms of cell injury, and its occurrence in all homoiothermic species, suggest that it fulfills a beneficial function. With respect to CRP this view is supported by the evolutionary conservation of structure and binding reactivity of the CRP

family. CRP of all species binds to phosphorylcholine (PC) residues in the presence of calcium ions and PC-binding proteins homologous with human CRP occur in many vertebrates, including fish, and even in an invertebrate, *Limulus polyphemus*, the horseshoe crab. A normal human plasma protein, known as serum amyloid P component (SAP) because the molecule is pentagonal in shape and because it is laid down in amyloid deposits, closely resembles CRP in structure with 60–70 per cent homology of amino acid sequence. Homologues of human SAP are also present in all vertebrate classes.

A serious pathological complication of a sustained acute phase response, due to persistent infection or inflammation, is the deposition of amyloid A (AA), an extracellular accumulation of protein fibrils consisting of peptide subunits arranged in an anti-parallel β -pleated sheet. The protein is derived from a serum precursor (SAA) which is an apolipoprotein of high-density lipoprotein produced in the acute phase. The trigger molecule initiating synthesis of SAA and other acute phase proteins by liver cells is apparently interleukin-1 (IL1), a peptide product of macrophages. IL1 also activates T lymphocytes and is a pyrogen. Its production can be initiated either by direct stimulation of macrophages, for example, with bacterial lipopolysaccharide, or indirectly by mediator(s) released from damaged tissue cells, possibly prostaglandins. The mechanism by which liver cells increase the abundance of mRNA for acute phase proteins in order to provide for their increased synthesis is not yet known. However, the genes for SAA

and for α_1 -acid glycoprotein, another acute phase reactant, have now been cloned and this may provide information on control of synthesis as well as on the structure, functions and evolution of the proteins themselves.

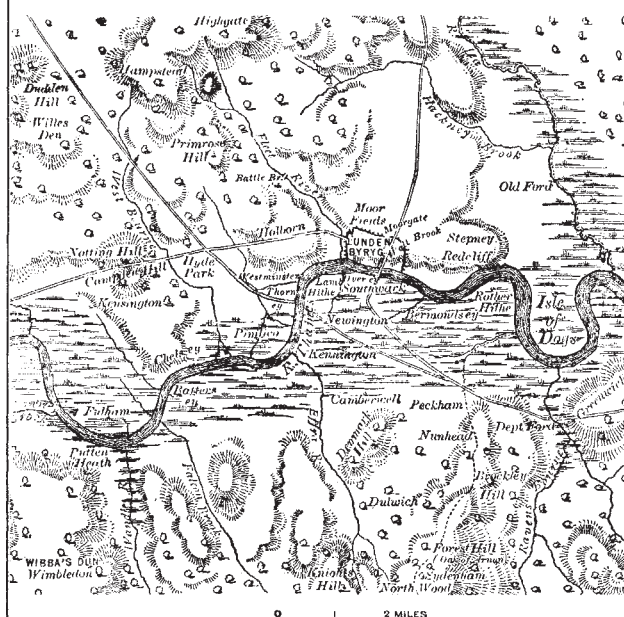
The normal *in vivo* functions of CRP, SAP and SAA are not yet known. Presumably, for CRP and SAP these are related in some way to their strictly conserved ligand-binding properties. CRP binds preferentially to damaged rather than intact autologous cell membranes and having done so, human CRP activates the complement system; part of its role may be to elicit the inflammation required for resolution and repair. CRP also binds to extrinsic ligands and can thus enhance resistance, for example to pneumococcal infection in mice. In addition to being a normal plasma protein and a constituent of amyloid deposits, SAP or a closely related protein is also a normal tissue protein. It is found in glomerular basement membrane and in association with elastic fibre microfibrils throughout the body. Its role in tissues is not known but it is of interest that aggregated SAP selectively binds fibronectin from whole serum.

Clinical measurements of serum CRP, which, like basic studies of CRP itself, suffered a long period of decline, are now being treated with fresh enthusiasm. Such measurements are useful in screening for organic disease and in monitoring the activity of many inflammatory or necrosing disorders, and are a sensitive test for infection. Although the acute phase response is a non-specific manifestation of tissue injury its precise quantification can provide a valuable aid to the clinician at the bedside. □

*'C-reactive protein and the plasma protein response to tissue injury', organized by I. Kushner, H. Gewurz & J. E. Volanakis and held at the Barbizon-Plaza Hotel, New York, 21–23 September 1981 under the auspices of the New York Academy of Sciences. Proceedings to be published in the *Annals of the New York Academy of Sciences*, 1982.

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100 years ago



From *Nature* 25, 2 March, 418, 1882.

"Nowhere has the hand of man moulded ground into shapes more strangely contrasted with its natural form than on the site of London. Even as late as the time of Caesar the soil which a large part of it covers can have been little but a vast morass. Below Fulham the river stretched at high tide over the ground that lies on either side of its present channel from the rise of Kensington and Hyde Park to the opposite shores of Peckham and Camberwell. All Pimlico and Westminster to the north, to the south all Battersea and Lambeth, all Newington and Kennington, all Bermondsey and Rotherhithe, formed a vast lagoon. Near the point where the Lea poured its waters into the Thames, a traveller who was mounting the Thames from the sea saw the first dry land to which his bark could steer. The spot was in fact the extremity of a low line of rising ground which was thrown out from the heights of Hampstead that border the river valley to the north, and which passed over the sites of our Hyde Park and Holborn to thrust itself on the east into the great morass.

The International Conference on Venus

After Pioneer—good science, bad news

OVER the last few years an intense study of the planet Venus has been undertaken following its exploration by spacecraft from the US and the USSR. At a recent meeting* in Palo Alto, every aspect of the planet was discussed. In the six articles that follow, contributors at that meeting survey a selection of the topics covered.

Our nearest planetary neighbour (its orbit has a radius seven-tenths that of the Earth), Venus has a size and mean density only slightly smaller than our own. Regrettably, even the best optical telescopes reveal a disk with an absence of surface features owing to the brightly reflecting clouds that shroud the planet.

It required high-sensitivity radar techniques to pierce the clouds and show that Venus rotates on its axis once every 243 days. Unlike all the other planets except Uranus, this rotation is in the opposite sense to the planet's orbital motion. In fact, this rotation rate ensures that approximately the same face of Venus is turned towards the Earth when the two planets are closest to each other in their orbits. This implies that during the history of the Solar System, Venus has become locked into a rotational near-resonance by the Earth's gravitational influence; Venus must necessarily be axially asymmetric in its mass distribution.

Spectroscopy revealed that the atmosphere consisted mainly of carbon dioxide and that the clouds contained sulphuric acid, while the radar measurements revealed poorly defined large-scale surface features. Several direct measurements of surface conditions were provided by Soviet spacecraft. In 1975 Venera 9 and 10 provided the only surface photographs so far obtained revealing a boulder-strewn terrain. The surface temperature was found to be over 400 °C.

Our perception of Venus underwent a revolution in 1978, however, when several spacecraft reached the planet. One was the Pioneer multiprobe mission, whose five spacecraft entered the atmosphere on 9 December and ceased to send data about two hours after entry. Four of the craft reached the surface, two on the nightside and two on the dayside. A parallel mission was the Pioneer Orbiter, which circles the planet and sends data still. In the same month that Pioneer arrived, two Soviet spacecraft, Venera 11 and 12, reached Venus, both landing a probe on the surface.

One question that was finally answered by Pioneer concerned Venus's very high surface temperature. Although closer to the Sun, the high reflectivity of the atmosphere should have resulted in a surface temperature lower than that of the Earth. Pioneer confirmed what had been suspected before — that a greenhouse effect was responsible. A carbon dioxide atmosphere would not have supplied the required blanketing effect owing to spectral 'windows' in the IR band. Pioneer showed, however, not only that enough sunlight reached the surface to fuel the effect but also that the small water (~0.1 per cent) and sulphur dioxide concentrations could produce enough absorption to close those spectral windows (see the article by Taylor).

Venus is sufficiently similar to the Earth and sufficiently different to provide a fascinating climatological laboratory. The planet's very slow rotation rate results in small Coriolis forces that might be predicted to cause less distinct latitudinal effects (such as zoning) than those found on the Earth, let alone the fast-rotating giant planet Jupiter. The absence of oceans releases fluid theoreticians from the complications of land-sea contrasts and corresponding latent heat budgets. Moreover, the planet's rotation axis is only 3° removed from the perpendicular to its orbital plane, compared with the Earth's 23°. Thus seasonal effects are virtually absent on the planet. Another difference arising from the slow rotation rate, of course, is the very slow passage of solar heating over the surface.

The picture that has emerged is discussed in detail in three of the following articles. Briefly, the temperature gradients from equator to poles are very small owing to an efficient single circulation cell in each hemisphere, which transports heat from the equator to the poles. In fact, the gradients are larger than expected. Moreover, the massive atmosphere (ninety times the mass of the Earth's), with its consequent high thermal inertia, had been expected to smooth out any longitudinal (day-night) thermal contrasts in the lower atmosphere. That this is not the case has been attributed to the action of eddy and wave transport.

The role of atmospheric waves is also thought to be crucial to understanding the high cloud velocities. The patterns are observed to super-rotate about the planet in four days, at a speed of about 100 m s⁻¹.

Above and below the three main cloud layers (which are found to concentrate at heights between 45 and 70 km) the atmosphere is relatively calm. Theorists have concluded that the high momentum is pumped into this layer by waves and eddies from the lower atmosphere, as discussed by Elson below.

The atmospheric circulation cells are concentrated at cloud heights, where much of the solar heat is absorbed. The clouds must play an important part in atmospheric energetics. It should be remembered, however, that these clouds are not the result of condensation from a vapour transported upwards by convection, as is often the case on Earth. (Pioneer found Venus's lower atmosphere to be surprisingly stable.) As Knollenberg discusses, they are the long-lived products of the atmospheric photochemistry of Venus. They have been compared with the urban 'brown clouds' which are only gradually removed from our atmosphere by rain.

Venus also differs from the Earth in that it appears to lack a magnetic field. This has important implications for the uppermost atmosphere. A strong magnetic field, as on Earth, would interact with the magnetic plasma (the solar wind) that impinges on all the planets, and deflect it. On Venus the ionized upper atmosphere on the dayside faces the wind unprotected, with the result that energy from solar disturbances can be more directly dumped from interplanetary space into the upper atmosphere. Relevant processes are discussed in more detail in the articles by Nagy and Brace and by Cloutier and Russell.

The orbiting radar of Pioneer has mapped the surface topography. These observations, combined with measurements of the planet's gravitational field, have led to the conclusion that Venus's crust is considerably thicker than the Earth's and is not broken up into 'plates' (see article by McGill). The heat from the planetary interior appears to be reaching the surface primarily beneath two elevated regions, the Beta Regio and the Aphrodite Terra highlands. The detection of volcanic land forms and radar signatures comparable with those observed from lava flows on Earth has led some to believe that these regions are currently volcanically active. This belief is supported by the discovery that the sources of lightning on Venus appear to be concentrated in these two 'volcanic' regions, suggesting that arcing

*The International Conference on the 'Venus Environment' was held in Palo Alto, California on 1-6 November 1981.

within volcanic dust clouds (as happened in the Mt St Helens eruption) may be occurring.

One of the most surprising results from the Pioneer and Venera measurements was that the abundances of non-radiogenic rare gases such as ^{36}Ar were much higher than on the Earth. In fact, the values decrease in order-of-magnitude steps from Venus to Earth to Mars. The atmospheric abundances of these non-reactive gases result from three possible processes: (1) accretion during planetary formation from the solar nebula; (2) accretion from planetary bombardment by asteroids and comets; and (3) outgassing from planetary interiors. None of these processes would be expected to result in the observed distribution. Resolution of this problem is some way off, and is not helped by the fact

that different rare gases appear to be telling different stories.

What of the future? the next major advances in Venus science will hopefully come from the USSR. Two more Venera craft were launched at the end of last year, equipped with landers aimed at the Beta Regio area. Two further spacecraft are expected to be launched in 1984 or 1985, which will release two landers onto Venus before proceeding to Comet Halley. Meanwhile, the reduced NASA budget is impeding the further analysis of planetary data. In view of the stimulating results from Venus, such a situation can most politely be described as tragic.

Philip Campbell

Philip Campbell is Physical Sciences Editor of Nature.

Geology and geophysics

from George E. McGill

EVEN though Venus is the most Earth-like object in the Solar System, there are important geological and geophysical differences, the most notable being the high pressure and temperature of the atmosphere (~ 96 bar and 740K at the median elevation)¹, and the very low abundance of water². These characteristics imply that at shallow depths, Venus is hotter and drier than Earth. Nevertheless, recent models of thermal evolution predict temperatures very similar to those on Earth in mantle regions subject to solid-state convection³⁻⁵. The high temperature of the shallow interior implies a more buoyant lithosphere than on Earth for several reasons^{6,7}, some of which depend critically on the per cent of bound H_2O in the upper mantle, a quantity that strongly influences

lithosphere thickness⁸ but which remains unknown.

Pioneer Venus radar altimetry has been carried out over 93 per cent of the surface. In contrast to the bimodal distribution of surface altitude of Earth, the Venus topographical spectrum is markedly unimodal (Fig. 1), with 60 per cent of the known surface within 500 m of the median radius⁹. The dual level of Earth topography results from (1) the contrasting densities of the two types of crustal rocks, continental and oceanic (the continental 'sial' and the denser oceanic 'sima' composed predominantly of silica and, respectively, aluminium and magnesium); (2) the Earth's plate tectonic activity, which continually creates thin, low-standing sima while sweeping thicker, higher-standing

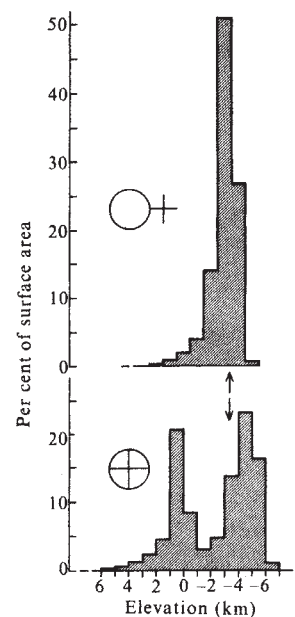


Fig. 1 Hypsographic histograms of Earth and Venus, in 1 km elevation intervals. The scale at the top is Earth elevation relative to sea level; the Venus histogram is matched to the Earth histogram at median elevations (arrows).

sial into clumps; and (3) the presence of oceans that trap most sediment close to the continents. If Venus has differentiated a significant amount of sial, then the strongly unimodal elevation spectrum implies that horizontal transport of the lithosphere due to plate tectonics, if present at all, must be even slower than the presumably very slow rates of erosion and sediment transport expected on a planet with no hydrologic cycle.

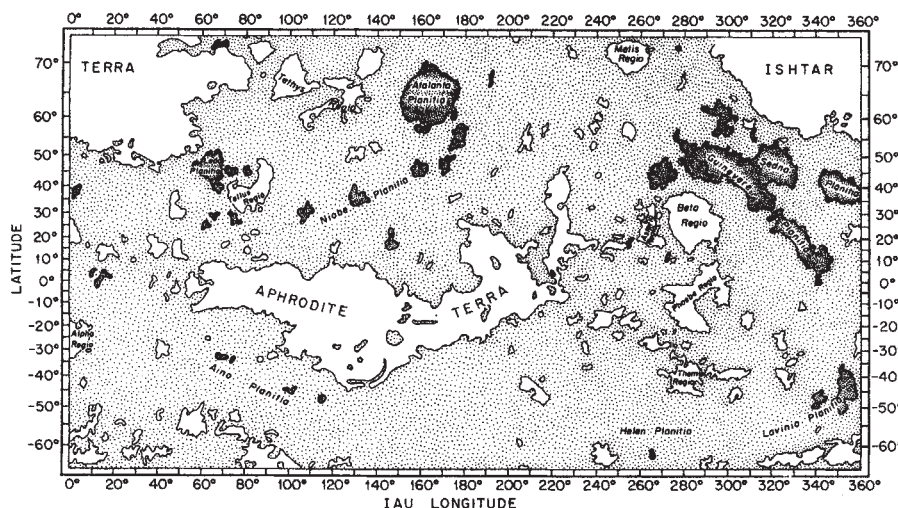
Crater-like forms occur on the venerean lowlands¹⁰; some of these may be of impact origin, some probably are volcanic, but most are enigmatic. The areally limited elevated regions include many tectonically interesting topographical features: large, straight to arcuate troughs up to 2,500 km long, round to irregular depressions, linear zones of 'pitted and hilly' terrain, and large massifs. Most of these features are associated with three regions: Aphrodite Terra, Ishtar Terra and Beta Regio (Fig. 2).

The largest elevated area is Aphrodite. In its eastern portion are several major arcuate troughs with flanking ridges⁹ and a moderately elevated terrain characterized by irregular depressions and hills (Fig. 3). The entire system of pitted and hilly terrain and troughs defines a linear zone that is radar rough at both centimetre and metre scales¹⁰ and which is almost certainly of tectonic origin¹¹⁻¹³.

Ishtar Terra includes a large plateau (Lakshmi Planum) flanked by narrow linear mountain belts along its western and northwestern margins, and by a large massif (Maxwell Montes) along its eastern

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Fig. 2 Major topographical regions of Venus. Elevations >1 km above median clear; elevations within 1 km of median in light stipple; elevations >1 km below median in heavy stipple. Mercator projection greatly exaggerates the size of Ishtar Terra, which actually includes about half as much area as Aphrodite Terra.



margin which contains a point 11 km above median — the highest so far discovered on Venus. On the flanks of this massif are several depressions, including a circular region smoother at centimetre scale than its surroundings¹⁴. The depressions are probably volcanic in origin¹⁰. Two larger but much more irregular depressions occur on Lakshmi Planum. Their obvious non-circularity and their association with large 'canyons' extending into the plateau from its southern rim constitute strong evidence that they are of tectonic or volcanic origin rather than due to meteorite impact^{12,13}. The general association of a large plateau, volcanic and tectonic depressions, and linear mountain belts has led some workers to suggest that Ishtar is the best candidate for a true continent on Venus^{4,5}.

Beta Regio appears to be a domical uplift with a medial rift, and with at least one large shield volcano built on it^{14,15}. This association, together with some additional topographical details, lead naturally to the inference that the Beta Regio area is analogous to a continental rift system, such as in East Africa^{12,13,16}.

Despite the presence of some landforms suggesting active tectonics, there is no evidence of a global system of spreading ridges and subduction trenches characteristic of Earth's plate tectonics even though the resolution of the Pioneer Venus altimetry was adequate to resolve them had

they been present¹⁷. If the tectonic features described above are assumed to be part of a smaller-scale plate tectonics system, it can be shown that such a system would be grossly inefficient in removing heat from the interior of Venus^{4,5,18}.

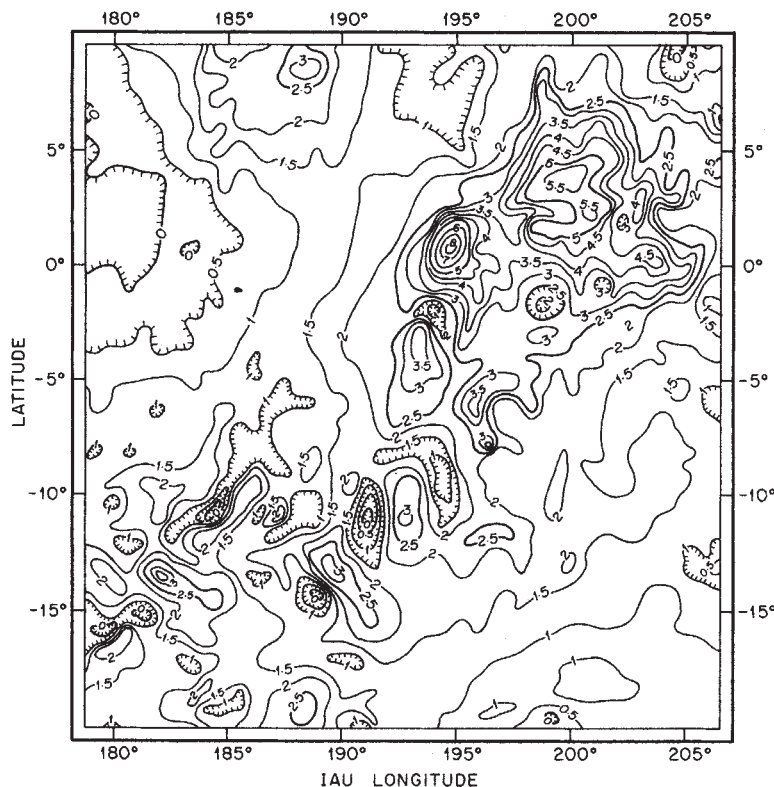
Surface properties and processes also provide some limited data bearing on the geological evolution of Venus. Igneous rocks on Venus are expected to contain minerals that are essentially identical with those found in common rocks on Earth (possibly excepting hydrous phases)^{12,13}. These minerals should be chemically weathered by the venerean atmosphere, particularly at high elevations¹⁹. Aeolian transport of weathering products can result in atmosphere/rock chemical cycles because of the large variations in atmospheric pressure and temperature (~ 60 bar and ~ 110 K) that accompany the >13 km relief on Venus^{1,9}. Expected threshold friction speeds appear fully sufficient to erode and transport particulates given the ~ 1 m s⁻¹ free-stream wind velocities measured by Venera and Pioneer probes near the surface^{12,13,20-22}.

Earth-based and Pioneer radar determinations of the Fresnel reflection coefficient indicate that the surface of Venus cannot be covered by a thick (more than a few centimetres), areally extensive layer of porous regolith or aeolian sediment^{12,13}. It is possible, however, that

aeolian sediment is cemented by chemical reaction with the atmosphere, a process that would increase its bulk density and render it indistinguishable (to radar) from slightly weathered igneous rocks. Very high reflectivities that imply densities consistent with fresh mafic or ultramafic igneous rocks occur in only a few areas of high elevation, especially the southern part of Beta Regio²³. Presumably these areas are very young, or they are sites of active erosion.

Although both topographical data and thermal modelling predict that an Earth-like system of plate tectonics is not present on Venus now, the abundance of ⁴⁰Ar in the atmosphere of Venus² suggests that the overall rate of tectonic and volcanic activity during its evolution has been only a little below that of Earth. Moreover, long-wavelength gravity anomalies coincide in position with major topographical features, a correlation that, considering the hot lithosphere, suggests dynamic mantle support of the topography^{4,5,18}. How then is heat lost from the interior of Venus? Phillips (Lunar and Planetary Institute) suggests a tectonic style similar to intraplate, hot-spot tectonics on Earth^{4,5}. Perhaps Venus is characterized by a global system of domes, local rifts and active volcanic centres, with specific elements developing and decaying in concert with changes in the mantle flow patterns responsible for them, implying that little or none of the modern topography of Venus has survived from primordial times. This model suggests that modern Venus may well be tectonically similar to Archaean Earth. □

Fig. 3 'Pitted and hilly' terrain in eastern Aphrodite Terra. This terrain is much rougher to radar at both centimetre and metre scales than are the lower regions in the north-west and south-east corners of the map. Contour interval 0.5 km; datum is median radius.



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Structure and energetics of the atmosphere

from F. W. Taylor

FOR many years now, the key question about the structure of the atmosphere of Venus, and the mechanisms that shape it, has been the origin of the high surface temperatures and pressures (over 750K and nearly 100 bar respectively). It is now reasonably certain that the atmospheric 'greenhouse effect', whereby incoming short-wavelength solar radiation penetrates the clouds more efficiently than outgoing long-wavelength thermal radiation, traps enough heat to account for the severe conditions in the lower atmosphere. The wealth of new data from the recent Pioneer Venus and Venera missions has placed calculations of the greenhouse model on a much firmer footing than previously, with measurements of the amount of solar heating at different altitudes, the vertical distribution of cloud opacity and the total IR flux escaping from the top of the atmosphere.

About 2.5 per cent of the energy from the Sun intersected by Venus reaches all the way to the surface. About 80 per cent is reflected back into space by the very reflective sulphuric acid clouds without being absorbed by the planet at all. Of the remainder, about half is absorbed above 60 km altitude (mostly near the very top of the clouds) and the rest below. It is somewhat disturbing that the best calculations of the thermal component of the 'greenhouse' still allow about 200 W m^{-2} to escape from the top of the atmosphere on average, whereas only 158 W m^{-2} was observed by orbiting IR sensors. This suggests that there is a fairly important, but still unidentified, source of opacity in the clouds. However, the relatively low actual flux out makes it easier to 'balance' the greenhouse calculations at the required high surface temperatures. A further unresolved problem is that albedo (or reflectivity) measurements from the Earth and from space indicate that Venus keeps, on average, only about 145 W m^{-2} of the flux from the Sun, raising questions about where the other 10 per cent or so comes from. The albedo is probably less than the present best value of 0.80 indicated by a preliminary analysis of the data, and it is being re-examined.

The most spectacular new features emerging from the recent rash of exploration lie in the atmosphere at the cloud tops and above. At these levels the radiative and dynamical time constants are less than the venusian day and interesting diurnal (day-night) and seasonal (equator-pole) structure has emerged, much of it poorly understood. At the highest levels, in the part of the atmosphere called the exosphere (Fig.1), the temperature varies from 300K

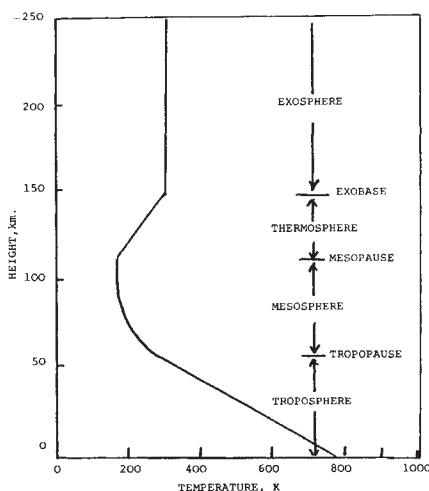


Fig.1 Nomenclature for the different atmospheric regions on Venus, based on the mean temperature structure by analogy with Earth. The thermosphere and exosphere are also known collectively as the 'upper' atmosphere, the mesosphere and troposphere as the 'middle' and 'lower' atmosphere respectively.

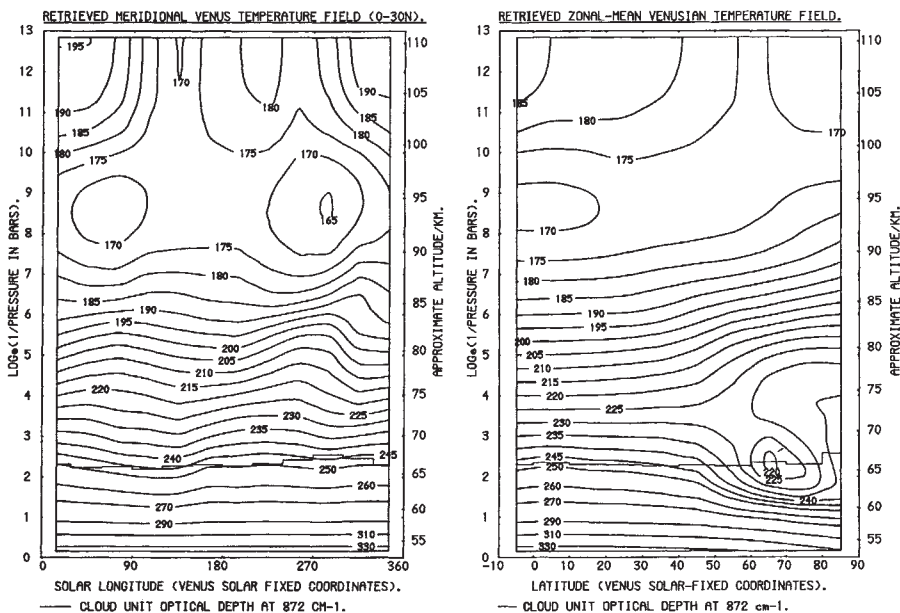
on the dayside (cooler than predicted by models) to a surprisingly low 100K on the nightside. It is very difficult to find cooling mechanisms efficient enough at these low pressures to explain the latter at all, and the very sharp drop which is observed across the terminator enhances the problem. At lower levels near the mesopause (Fig.1), the temperature distribution is more nearly as expected, with 'warm' air (185K) near local noon, a 'cool' (170K) nightside and an

'intermediate' (175K) pole. However, the simple pattern is upset by a 'hot spot' of 185K near local midnight, where solar heating is a minimum. This is thought to be produced dynamically, perhaps by compressional heating in the descending branch of a symmetric sub-solar to anti-solar circulation pattern. A little below the mesopause level, the distribution of temperature alters radically. At levels near 90 km altitude, the sub-solar point is the coolest on the planet and the poles are warmest. This remarkable state of affairs remains largely unexplained, although theories abound. These include absorption of solar radiation in a 'polar cap' of mesospheric haze, a 'hotplate' effect due to depression at the poles of the main cloud to warmer levels, and again dynamical heating, this time in the descending branch of a hemispherical Hadley-type cell. The reversed equator to pole temperature gradient persists nearly down to the cloud tops (Fig.2).

An interesting feature of any atmosphere is its response to the diurnal cycle of solar heating — the thermal tides. Instead of the familiar wavenumber one fluctuation of the Earth's atmosphere (single maximum and minimum temperatures around an equatorial circumference), Venus surprises with a wavenumber two tide. Near the cloud tops, temperatures peak some 5K above the mean value in the morning and again in the second half of the night, with minima in the afternoon and post-duck quadrants. Why does Venus respond to predominantly wavenumber one solar forcing with a wavenumber two tide? Theorists are beginning to develop tidal models which are outlined below by Elson.

Even more obscure are the mechanisms which give rise to the two most pro-

Fig.2 (left) An equatorial cross-section through the mean atmospheric temperature field on Venus, from Pioneer Venus IR soundings. 0° solar longitude is the local noon, 90° the morning terminator and so on (remembering that Venus rotates in the retrograde sense). The stepped line near the bottom of the figure is the height of the main cloud top as a function of local time on Venus. Note the thermal tides. **Fig.3** (right) As Fig.2, but an equator to pole cross-section (0° = equator, 90° = N pole). Note the polar warming and the circumpolar collar.



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minent phenomena of Venus's atmosphere, the polar dipole and the circumpolar collar. The true nature of these was hidden from Earth-based observers for decades because they occur at such high latitudes (65–70° and 80–90° respectively) as to be foreshortened beyond recognition to all but the polar-orbiting Pioneer Venus Orbiter. They appear in PVO IR images as a very cold, nearly circular feature about 5,000 km in diameter centred on the pole (the collar) and two hot features, thought to be holes in the otherwise ubiquitous cloud cover, about 1,000 km either side of the pole and rotating around it (the dipole; see *Nature* 279, 613; 1979). A meridional cross-section of the temperature structure of the middle and upper atmospheres (Fig. 3) reveals the collar as an intense, cold planetary-scale wave of long duration. It

has been speculated that it is a disturbance produced at the boundary between the 'solid body' rotation of the atmosphere, which is observed in cloud-tracked winds and latitudes equatorwards of the collar, and the regime polewards in which the atmosphere accelerates in the zonal direction as it flows towards the pole, in an attempt to conserve angular momentum. This acceleration is evident in the high rotation rate (every 2.5 to 3 days) of the dipole at the centre of the collar. The dipole is the eye at the centre of the polar vortex on Venus, remarkable for its 'double' appearance. Two clearings in the polar cloud orbit around the pole, rather than the expected one at its centre. The dipole itself is perhaps the biggest mystery of Venus' atmosphere, and a prime target for future missions to the planet. □

may reappear above 100 km and, as was discussed by Mayr, it seems to be required at altitudes above 150 km to explain Pioneer observations.

Atmospheric thermal tides, temperature and wind perturbations caused by solar heating of the atmosphere were addressed by several speakers. Elson discussed them from an observational and diagnostic point of view, suggesting that the temperatures observed by the Pioneer spacecraft are difficult to reconcile with our terrestrial experience, and that model calculations indicate that the tidal winds are probably small in amplitude and dominated by dissipative forces. Pechmann (California Institute of Technology) described theoretical calculations of tidal winds and temperatures using a model. Her results suggest that the tidal response of the atmosphere strongly depends on the mean (zonal and time averaged) zonal wind and static stability. A similar calculation of the tidal fields was made by Fels (Princeton University) but for a very different purpose. Assuming that the unusual observed warming at the poles has a non-radiative source, Fels chose to examine a dynamical forcing mechanism which relies on the interaction between tidally forced waves and the averaged flow whereby the tides force the averaged flow to descend in the polar regions. This descent produces warming at the pole due to adiabatic compression of the atmospheric gas as it moves down to higher pressures. To produce warmings as large as are observed, however, this mechanism must assume that the cloud level winds are much stronger than those observed.

Tides were not the only type of waves to be discussed during the session. Apt (Jet Propulsion Laboratory) reported the presence of waves with periods of 5.3 and 2.9 Earth days over the entire Northern Hemisphere between the cloud tops at about 65 km and 90 km. These waves are thermal perturbations observed at IR wavelengths. Del Genio (NASA Goddard Institute for Space Studies) reported the existence of a 5.2 day wave from UV observations. Perhaps there is a temperature-sensitive UV absorber present. In the area of theoretical wave calculations, a presentation was made concerning results produced by a general circulation model of the type used for comprehensive terrestrial studies. Young (NASA Ames Research Center) described calculations of baroclinically unstable waves whose terrestrial counterparts are responsible for most of what is called weather. As is the case on the Earth, these waves are caused in part by the vertical shear in the zonal wind. Despite this similarity, differences in the rotation rates and atmospheric structures of the two planets appear to produce different wave characteristics. □

Dynamics of the atmosphere

from Lee S. Elson

ATTEMPTS to explain the observation that the entire atmosphere of Venus moves in the same retrograde (westward) direction provided one important focus of the conference. Schubert (University of California, Los Angeles, and see refs 1, 2) described the atmospheric circulation as it is now known from the Pioneer Venus and the Soviet Venera spacecraft. The atmosphere moves westwards at average speeds of up to about 110 m s⁻¹ and there is a meridional (north/south) cloud level cell analogous to the terrestrial Hadley cell. As shown in Fig. 1 (from Schubert's paper), there is considerable variation in this flow with time. Therefore, the term '4 day wind' used to describe the periodicity of UV features advected by the retrograde winds is a misnomer, because the wind speed corresponds more closely to a 5 day period.

Several suggestions for the possible causal mechanisms of this flow were made in the context of comparative planetary meteorology. Mayr (Goddard Space Flight Center) and Williams (Princeton University) considered the role of meridional cells, viscosity and solar heating of the atmosphere in producing zonal (east/west) jets on Jupiter, Saturn and Venus. Their conclusions and the discussion with the audience indicated that one might expect such jets on Venus if several mechanisms operated simultaneously. However, model calculations are still unable to reproduce exactly the observed flow while simultaneously satisfying heat and momentum conservation constraints. Two papers dealing with the zonal jet above the clouds were also presented. Elson (Jet Propulsion

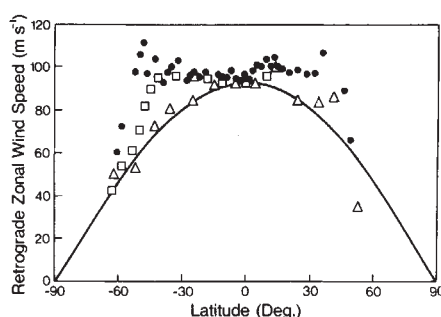


Fig. 1 Mean cloudtop westward wind as a function of latitude. Mariner 10 data from 1974 (filled dots) show different structure from that of Pioneer Venus as shown by the open symbols representing UV data presented by Del Genio.

Laboratory) described evidence³ that the zonal jet dies off with increasing altitude so that it has disappeared entirely above about 80 km altitude (see Fig. 2). The jet

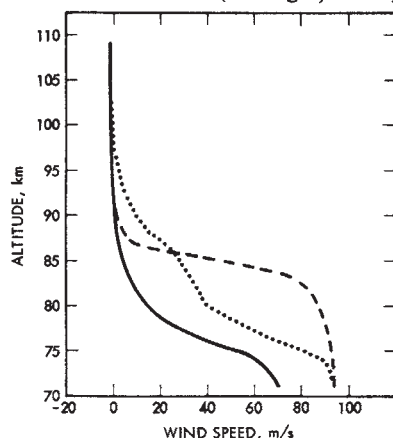


Fig. 2 Mean westward wind as a function of altitude above the cloud tops. The solid curve is the best estimate based on a model⁴ and the other curves are the results of a study of the model sensitivity to input assumptions.

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Clouds and hazes

from R.G. Knollenberg

THE Venus cloud and haze system summarized in Table 1 is planetary in nature with an enormous vertical extent of 50–60 km. The clouds are rather tenuous and were found to have an average visibility of about 1 km by early Venera nephelometer (particle backscatter photometer) measurements¹. The main cloud deck is at an altitude of 45–70 km and includes particles of various compositions². Upper, middle and lower cloud regions are easily defined by transition layers and changes in size properties. Haze layers overlie and underlie the main cloud deck.

Through an analysis of polarimetry data, Kawabata has shown that the upper submicron aerosol haze has an average optical depth of about 1 in its most dense polar regions where it is a distinct layer above the main cloud deck³. In the equatorial and mid-latitude regions, it is about one-tenth as dense and is mixed with 2 μm diameter monodispersed H_2SO_4 cloud droplets at the top of the main cloud deck. The haze particles also have optical and physiochemical properties consistent with H_2SO_4 .

Regent and Blamont's nephelometer data show a sharp base for the main cloud deck at 47–50 km at four different Pioneer Venus entry sites but thin filaments are also evident below the nominal cloud base⁴. Knollenberg and Hunten have shown that the size distribution is generally multimodal throughout the main cloud deck, which has an optical depth of 20–30 (ref. 5). There are three size modes identified as mode 1 aerosol, which varies in composition with altitude; mode 2 H_2SO_4 droplets; and mode 3, which appears to include H_2SO_4 droplets and perhaps crystals of unknown composition. Below the main cloud deck, small haze particles of low number density extend down to approximately 30 km, where all particles essentially disappear.

Mode 1 is highly variable in all cloud regions and has been characterized as a background aerosol population with high number density but little optical depth except in the UV. Toon has shown that, in the upper cloud region, the mode 1 aerosols have a higher refractive index and seem to be rich in sulphur compounds⁶. In the lower cloud region, H_2SO_4 appears more plausible. Below the cloud deck, the mode 1 aerosols, which comprise the lower thin haze, appear to be desiccation products (cores) of cloud particle growth and decay.

The mode 2 H_2SO_4 droplets are probably several months old in the upper cloud region unless vertical exchange is faster than suspected, but perhaps only

hours old in the lower cloud region. At upper altitudes, H_2SO_4 droplets are likely to be contaminated after scavenging aerosol over their long lifetime. Near cloud top, H_2SO_4 vapour should be sufficiently supersaturated to support a bimodal population; that is, small droplets can nucleate and grow even in the presence of an existing larger droplet population. The mode 2 H_2SO_4 droplets nucleate and grow in the upper cloud region. Knollenberg and Hunten observe a gradual increase in number density descending through the main cloud deck. The mode regenerates through condensation in the lower layers. Its size distribution is exceedingly narrow at any one altitude — growth models of Turco and Toon indicate that dominant but competitive droplet diffusional growth can explain this⁷. The mode 2 distribution is uniform over most of the planet and is indicative of its extreme stability.

Analysis of particle image data by Knollenberg and Hunten showed the largest mode 3 particles to be asymmetric and suggested a crystalline species interpretation. Toon has shown that the radiative flux and single particle optical data are consistent without a crystalline species⁸ and favours H_2SO_4 droplets. Additional measurements will probably be required to resolve this controversy.

The entire Venus cloud system has an optical depth of 20–35 at visible wavelengths. Taylor reports a radiometric albedo of 0.77–0.82, increasing from equator to pole⁹. His IR radiometer data have revealed a strong dipole circulation near the poles. Tomasko finds single scattering albedoes ranging from a low of 0.995 in the upper cloud region to 0.999 in the lower cloud region¹⁰. For most of the radiometric spectrum the planet appears featureless; however, fascinating contrasts are observed in the UV via partially understood absorption processes. Pollack and Esposito find that sulphur dioxide is responsible for absorption at short UV wavelengths, but the identity of the absorber visible from Earth in the near-UV

remains unknown^{11,12}. Particulate sulphur (mode 1) appears to be a possible choice for secondary absorber. The related question of how the physical and/or chemical processes at the cloud top act to bring about the horizontal variability of these absorbing constituents is still a puzzle.

The main cloud deck is embedded in the zonal circulation at altitudes of greatest wind velocity and vertical wind shear. The clouds are all of stratiform morphology. Cloud particle growth is not strongly influenced by either the large-scale circulation or the latent heat released during condensation. The clouds are most strongly influenced by radiative exchanges. Because of the temperature extremes, there is a rather large scale of H_2SO_4 droplet growth times ranging from months in the upper hazes to hours in the lower cloud region. There is little evidence for the existence of large precipitation particles. Known processes for the formation of the lightning observed by Ksanfomaliti and Scarf^{13,14} require both. If cloud processes generate the lightning, then much larger undetected particles must exist. Scarf has suggested that the Venus lightning is phase-locked with the surface and may be solely the result of volcanic activity¹⁵. □

Table 1 Summary of Venus cloud and haze properties

Region	Altitude (km)	Temperature (K)	Optical depth τ	Av. no. density (per cm^3)	Mean diameter (μm)	Composition
Upper haze	70–90	225–190	0.2–1.0	500	0.4	H_2SO_4
Upper cloud	56.5–70	286–225	6.0–8.0	(1)–1,500 (2)–50	Bimodal 0.4, 2.0	H_2SO_4 + sulphur
Middle cloud	50.5–56.5	345–286	8.0–10	(1)–300 (2)–50 (3)–10	Trimodal 0.3, 2.5, 7.0	H_2SO_4 + crystals (?)
Lower cloud	47.5–50.5	367–345	6.0–12	(1)–1,200 (2)–50 (3)–50	Trimodal 0.4, 2.0, 8.0	H_2SO_4 + crystals (?)
Lower haze	31–47.5	482–367	0.1–0.2	2–20	0.2	H_2SO_4 + aerosol
Pre-cloud layers	46, 47.5	378, 367	0.05, 0.1	50, 150	Bimodal 0.3, 2.0	H_2SO_4 + aerosol

R.G. Knollenberg is President of Particle Measuring Systems Inc., 1855 South 57th Court, Boulder, Colorado 80301.

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Structure and dynamics of the ionosphere

from A.F. Nagy and L.H. Brace

A general view of the ionospheric structure of Venus and of the major processes operating there are shown in the figure. Solar extreme ultraviolet (EUV) radiation falling on the thermosphere creates the daytime ionosphere, which is in photochemical equilibrium near its peak at about 142 km, where the major ion is O_2^+ ; transport processes become dominant above about 200 km, where the major ion is O^+ . The upper ionosphere is in near diffusive equilibrium much of the time, although ion transport to the nightside and the loss of plasma through the ionopause frequently cause departures from diffusive equilibrium at high altitudes and in the region near the terminator. A comparison of calculated and measured ion composition indicates that we have achieved a first-order understanding of the very complex photochemical processes involved. Recent suggestions for the inclusion of a large number of specific reactions involving metastable species have even further narrowed the differences between the models and the experimental results. The dayside ionosphere is heated internally by solar EUV-generated photoelectrons and heated from above by the downward conduction of solar wind energy deposited at the ionopause. The ion gas also receives heat from exothermic chemical reactions

and Joule or frictional heating.

The day-to-night plasma pressure gradient across the terminator drives a nightward flow of ions, usually at supersonic velocities; some of these ions subside to contribute to the formation of the nightside ionosphere. The great variability of the nightside ionosphere is believed to arise from changes in the proportion of the flow which is lost to the wake. Even without the nightward ion flow, a nightside ionosphere would be formed by the precipitation of electrons with energies up to a few hundred electron volts, although the measured electron fluxes do not appear adequate to be the only source of the observed nightside ionization.

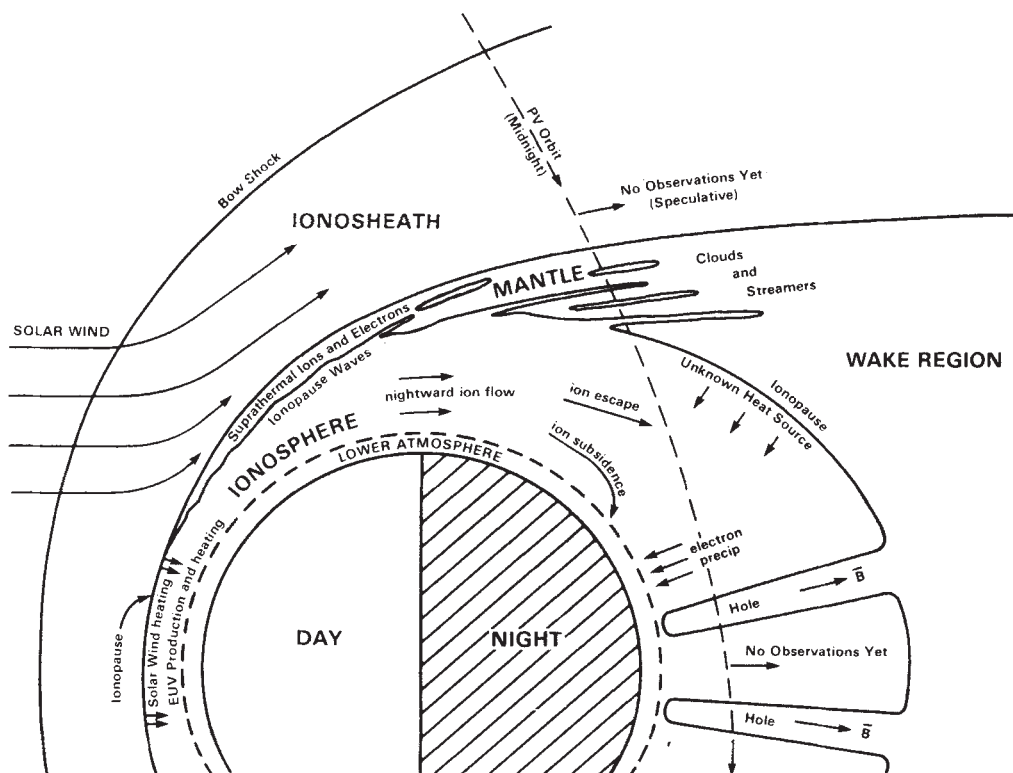
Large-scale radial holes or plasma depletions extend downwards to nearly the peak of ionization in the antisolar region. These holes, which occur in north-south pairs, enclose regions of strong radial magnetic fields that are believed to originate in the magnetotail of Venus. The holes or, more precisely, the radial magnetic fields act as a barrier to the flow of ionization into the antisolar region and may be related to the greatly elevated ion temperatures observed there. It has been suggested that the holes are formed by the removal of plasma by outward acceleration caused by magnetotail-generated electric fields.

The ionopause itself is a highly dynamic and complex surface which extends from an average altitude of 290 km at the subsolar point to about 1,000 km at the terminator. Its altitude varies from 200 to

more than 3,000 km on the nightside, though its height in the antisolar region will not be measured until further increases occur in the Venus orbiter periapsis altitude during the next few years. The increase in ionopause altitude from the subsolar point towards the terminator reflects the decrease of solar wind dynamic pressure with solar zenith angle. The ionopause altitude is highly variable, with a time constant of probably less than one hour. On the dayside, a near static balance between the solar wind and ionospheric plasma pressures is maintained, which results in an ionopause altitude variation which is inversely proportional to the wind pressure. The solar wind pressure is conveyed to the dayside ionosphere primarily by means of an enhanced magnetic field approximately parallel to the ionopause surface, although some of the solar wind plasma may interact directly with the ionospheric plasma.

Just above the ionopause is the mantle, a transition region between the ionospheric plasma and the flowing shocked solar wind plasma. A variety of solar wind interaction products are observed in the mantle, including suprathermal ions and electrons, ionospheric photoelectrons, enhanced plasma wave activity, and clouds or streamers of ionospheric plasma that appear to be swept downstream by ionosheath flow. These features are probably signatures of the plasma processes that form the ionopause and divert the solar wind about Venus; however the specific processes have not yet been identified. □

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Current views of the ionospheric structure of Venus, showing the major processes that are believed to be operating in the region. The scale of the ionosphere is increased by a factor of three relative to the ionosheath for clarity of presentation (reproduced from Brace, L.H. *et al.* *The Ionosphere of Venus: Observations and their Interpretation*, Venus, University of Arizona Press; 1982).

The solar wind interaction

from P.A. Cloutier and C.T. Russell

VENUS provides our only well documented example of the interaction of supersonic or, more correctly, supermagnetosonic magnetized plasma with an unmagnetized and highly-electrically conducting ionosphere. The Pioneer Venus observations confirm the zero-order picture derived from the earlier Venera and Mariner missions as sketched in Fig.1. A bow shock forms in front of the planet which heats, slows and deflects the flow around the ionosphere. The magnetic field is draped over the obstacle and increases in strength towards the planet while at the same time slipping around the planet as the plasma flows out of the plane of the figure. The physics by which this behaviour takes place is fairly well understood. The interesting physics, and that unique to Venus, at least so far, involves perturbations of this picture. These perturbations provided much of the controversy in the discussions of the solar wind interaction on the last day of the Venus conference.

Venus has a magnetic tail very like a comet. The Pioneer Venus magnetometer shows that behind Venus, in a region about 4 Venus radii across, the magnetic field is parallel to the solar wind flow and enhanced above interplanetary levels. Barnes (NASA Ames Research Center) and Intriligator (Carmel Research Center) reported that in the distant tail, interesting plasma effects are seen, including regions of depletion of solar wind plasma and regions of pick-up of ionospheric oxygen ions. Closer to the planet, Barnes observed the acceleration region for the oxygen pick-up. Near the terminator, the oxygen flow was weakest and fastest at high altitudes, growing in density and decreasing in speed as the planet was approached.

The source of this oxygen is presumably charge exchange and photoionization of the hot neutral oxygen exosphere at altitudes above the normal ionosphere. At high altitudes, this new cold plasma is accelerated either by the solar wind electric field or by magnetic stresses and escapes from the planet. At low altitudes, in the ionosphere, the magnetic and electric fields are weak and new ions remain bound by the planet's gravitational field. It is difficult to assess how important these processes are. Vaisberg and Zeleny (Space Research Institute, Moscow) have modelled mass loading of field lines due to photoionization in a cylindrical geometry but find that the flux of oxygen is too low. Perhaps charge exchange will provide the

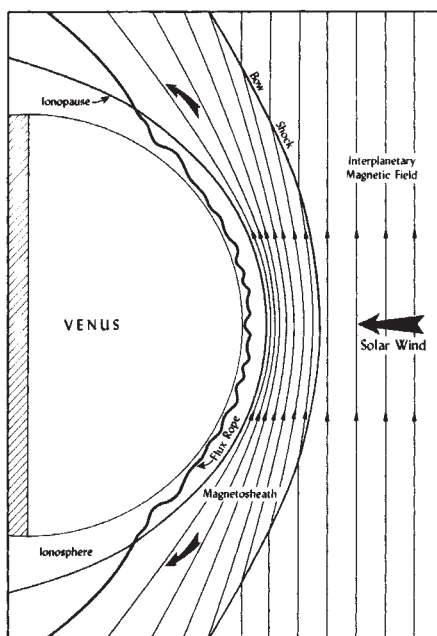
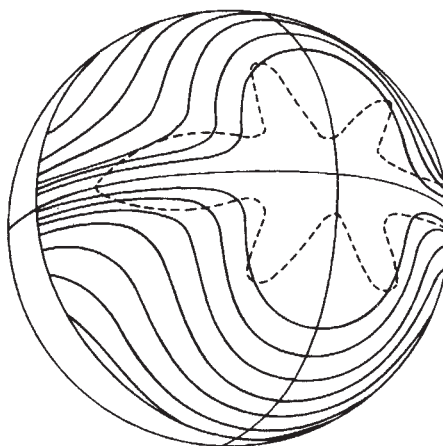


Fig. 1 Sketch of the interaction of the magnetized solar wind plasma with Venus's unmagnetized and highly-electrically conducting ionosphere.

missing flux but they prefer a source of anomalous ionization. Another indicator of the importance of these non-magnetohydrodynamic processes is the position (Slavin, UCLA, and Mihalov, NASA Ames Research Center) of bowshock. The shock is somewhat weaker and closer to Venus than would be expected if all the solar wind were deflected by the planet. Charge exchange could provide the requisite removal of momentum from the flow.

Fig. 2 Magnetic field line configuration on Venus's dayside (see the text). The dashed line shows the boundary separating regions stable and unstable to the Kelvin-Helmholtz instability.



Some of these phenomena were anticipated by a minority of scientists before the latest barrage of data from Pioneer Venus. Many, however, were not. Two unexpected phenomena were discussed by Brace (NASA Goddard Space Flight Center): plasma clouds, dense regions of cold plasma apparently detached from the ionosphere proper in the terminator region and ionospheric holes, regions of density depletion accompanied by radial magnetic field in the near midnight ionosphere. Another unexpected feature of the dayside ionosphere is the existence of magnetic flux ropes, twisted bundles of magnetic flux which often pervade the dayside ionosphere. An artist's conception of the occurrence of such a rope is given in Fig.1.

Flux ropes and other observed features of the dayside ionospheric configuration may be explained as resulting from induced convection within the ionosphere driven by the solar wind interaction. In a model proposed by Cloutier (Rice University) and his colleagues, pressure gradients and electric fields induced in the ionosphere drive an anti-sunward convection pattern which carries ionization and the draped interplanetary magnetic field towards the anti-solar point. In the model, velocity shear within the ionospheric convection pattern leads to flux rope formation due to the onset of the Kelvin-Helmholtz instability. Calculations of the ion convection patterns, the field geometry and the extent of the Kelvin-Helmholtz unstable regions reproduce many of the observed characteristics of the dayside Venus magnetic field behaviour, including the presence or absence of flux ropes, the occurrence of large magnetic fields at low altitudes, and the lack of coincidence of times between the ionopause and large magnetic gradients. The field line configuration is shown in Fig.2 by the solid lines, and the dashed contour shows the boundary between region stable and unstable to the Kelvin-Helmholtz instability. Flux rope formation may occur outside of the dashed contour.

The importance of the Kelvin-Helmholtz waves at the ionopause was discussed by Curtis (NASA Goddard Space Flight Center) who described the acceleration of ionosheath plasma by parallel electric fields arising from conversion of Kelvin-Helmholtz wave energy into a kinetic Alfvén wave. Curtis also showed that ions and electrons may be thermalized to high energies by high levels of plasma wave turbulence at Venus.

Finally, there are still some classical interaction problems to be solved: how, for example, does the flow close behind Venus (Knudsen, Lockheed), and is the interaction viscous rather than inviscid as preferred by Knudsen (Pérez-de-Tejada, Institute Geofísica, Mexico). In all there was a great deal of controversy and evidence of many more important principles to be uncovered in the solar wind-Venus interaction problem. □

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ARTICLES

Resolution of conglomerates by stereoselective habit modifications

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A method for resolution of conglomerates, based on morphological differences between the enantiomorphous crystals induced by tailor-made resolved impurities is described. The relationship between the crystal structure of the affected enantiomorph and the stereochemistry of the added impurity is analysed in the systems glutamic acid . HCl, asparagine . H₂O and threonine in the presence of L- or D-amino acids, and it is found that the additive binds stereoselectively at the surface of the enantiomer of the same absolute configuration, occupying the same site of the amino acid groups of the host molecule. The modified side chains perturb in a second stage the regular growth of the crystal in the directions along which the side chains of the host are hydrogen bonded.

IN 1848 Louis Pasteur found that racemic sodium ammonium tartrate crystallizes as two monohydrates of enantiomorphous habits¹. His separation of these two types of crystals and the observation that in solution they showed optical rotations identical to those of solutions of the separate enantiomers established the foundations of modern stereochemistry.

This crystallization behaviour might have escaped Pasteur's attention had not the enantiomeric crystals in this system been readily distinguishable visually: the 'laevo' and 'dextro' crystals have particular 'hemihedral' faces, which make the habits enantiomorphous². This experiment is remarkable because the combination of such 'spontaneous resolution' with visual distinguishability ('hemihedrism') is rare³, with less than a dozen examples having been recorded⁴.

Our recent discovery of a new and general method for kinetic resolution of conglomerates also allows the assignment of absolute configuration of chiral molecules^{5,6}. In connection with a study on generation and amplification of optical activity in prebiotic conditions⁷⁻⁹, we found that the crystallization of a conglomerate $\{R\} + \{S\}$ is influenced in a dramatic way by the presence of small amounts of a resolved impurity S' . Selective adsorption of the additive S' on the growing $\{S\}$ crystals, of similar stereochemistry, induces a substantial decrease in growth rate of these crystals, allowing their kinetic separation from the unaffected $\{R\}$ crystals. This same effect causes the $\{S\}$ crystals to undergo, during growth, morphological changes which can be extreme¹⁰⁻¹⁴ and which allow easy visual identification and separation of the two enantiomorphs scheme (1).

undergo spontaneous resolution but do not display hemihedrism³.

We describe here the morphological aspects of this selective adsorption process and discuss how they affect the action

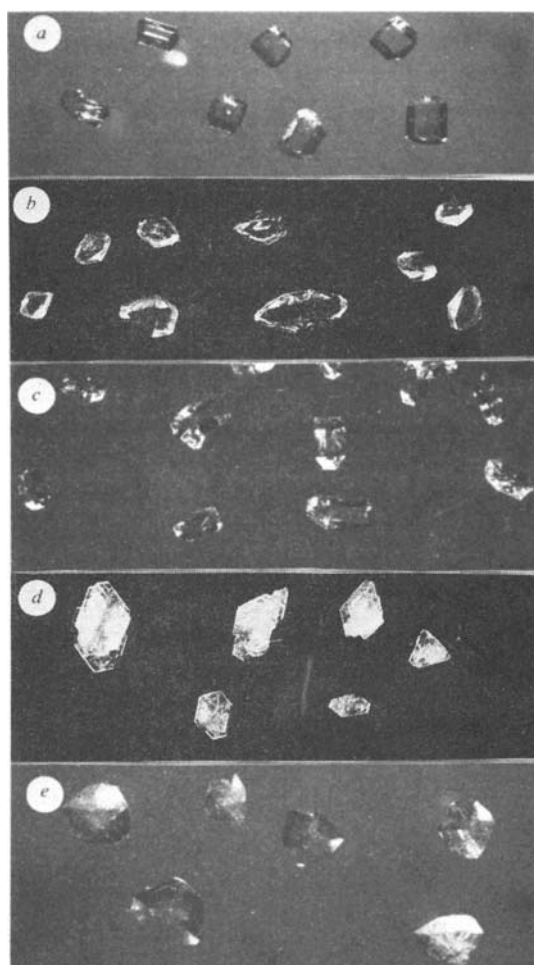
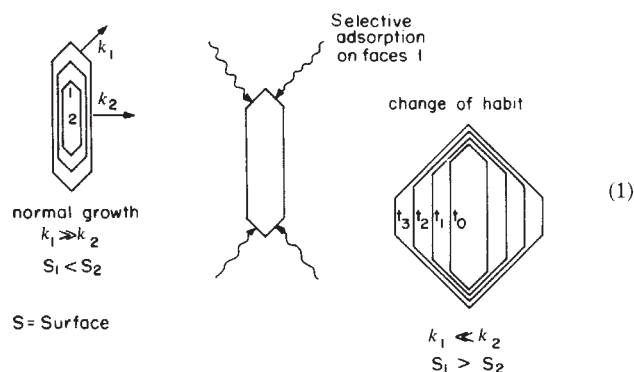


Fig. 1 Crystals of Asn . H₂O grown in presence of additives: a, none; b, D-Asn + (L) additive; c, L-Asn + L-Gln; d, L-Asn + L-Ser; e, L-Asn + L-Asp; f, L-Asn + L-Orn. In each case the crystals were obtained from D, L-Asn as a mixture of D-Asn displaying a morphology as in a, and L-Asn as in b, c, d or e, depending on the impurity.

This phenomenon offers the possibility of modifying the classical Pasteur experiment and of extending it to systems which

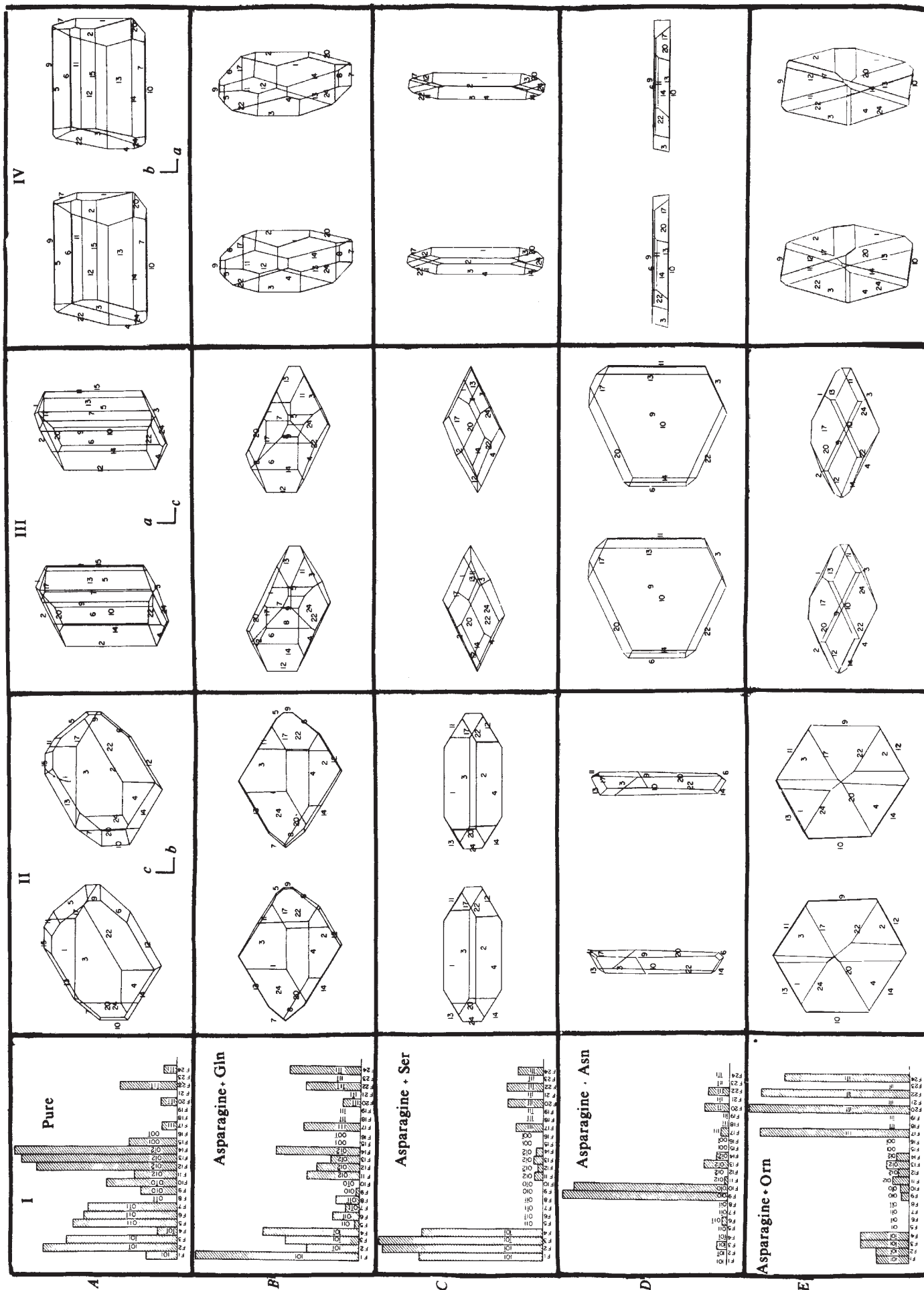


Fig. 2 A comparison between crystals of L-Asn . H₂O grown in presence of various additives: A, none; B, L-Asn + L-Gln; C, L-Asn + L-Ser; D, L-Asn + L-Asp; E, L-Asn + L-Orn. (I) are histograms describing the areas of the faces. The symbols F1, F2... correspond to faces 1, 2... denoted on the crystal pictures. The *hkl* Miller indices of these faces are shown on the histogram. (II), (III) and (IV) are computer-drawn stereographic pictures of the crystals viewed, respectively, along axes *a*, *b* and *c*, which form a right-handed system.

mechanism of the additive. We shall examine the stereochemical relationship between the additive and the crystal structure of the substrate, in order to exploit this knowledge for controlling crystal morphology. The conglomerates of D, L asparagine monohydrate, [D, L-Asn . H₂O] D, L-threonine [D, L-Thr] and D, L-glutamic acid hydrochloride [D, L-Glu . HCl] were selected for study.

Results

D, L-Asn crystallizes from neutral aqueous solutions in the form of a conglomerate [D + L] Asn . H₂O of space group P2₁2₁2₁ (refs 15–17). The crystals show a prismatic morphology with 18 well-developed faces (Figs 1a, 2A). We have found that resolved glutamic acid (Glu), aspartic acid (Asp), glutamine (Gln), serine (Ser), lysine (Lys), ornithine (Orn) and histidine (His) are effective in the kinetic resolution of racemic Asn; these same impurities were selected for morphological studies. Various batches of 100% supersaturated solutions (300 mg per 3 cm³) of D, L-Asn in water were prepared by cooling to room temperature hot filtered solutions, to which resolved impurity (L or D, 5–50 mg) had been added. Crystallization could be accomplished either spontaneously on standing for 1 or 2 days or on seeding with a powder of D, L crystals. In all cases crystals with a normal morphology precipitate first, but after a while (hours or days depending on the efficiency and concentration of the impurity) there appear crystals of distinctly different habit. The precipitates are then separated by decantation, dried and examined (Fig. 1). The crystals of changed and unchanged morphologies have identical internal structure, as checked by both powder and single crystal Weissenberg diffraction photographs, but consist of only L and D enantiomers, respectively, when L impurity is used (and vice versa when D impurity is used) (some typical results are reported in Table 1).

A decrease in concentration of the impurity (from 50 to 5 mg per 3 cm³) results in a progressive decrease in the modification of the affected enantiomer's morphology.

HPLC measurements, carried out in conditions in which D- and L-amino acids are resolved^{18,19}, confirmed that in the crystallization of pure L-Asn in the presence of racemic impurity (D', L'), L' is absorbed preferentially by the L crystals. Molar ratios of L'/D' from 6:1 for D, L-Glu to 30:1 for D, L-Asp were found²⁰.

To check the distribution of the D' and L' impurities in the L crystal grown in this way, single crystals (3–5 mg) were subjected to progressive dissolution in pure water, and the various fractions were analysed by HPLC. While the D' impurity was found only in the first dissolution fractions from L-Asn crystals, L' impurity was present in all fractions at approximately constant concentration. These results strongly support the idea that the impurity is situated only at the outer surface of the substrate crystals of opposite chirality, whereas it is occluded throughout the bulk of the crystal of the same chirality. Clearly the stereochemical similarity between L and L' is the key to the resolution process and to the morphological changes.

To try to clarify the mechanism of the adsorption process, we measured the dimensions, including the areas of all faces, of affected and unaffected crystals, and thus established which growth directions and the areas of which faces are modified by the occlusion of the impurity scheme (1). The crystal dimensions were determined on a diffractometer by measuring the perpendicular distance of each face from a convenient reference point²¹. These measurements were used to derive the coordinates of the bounding corners of the crystal from which the areas of the faces were calculated and the stereographic pictures of the crystals drawn.

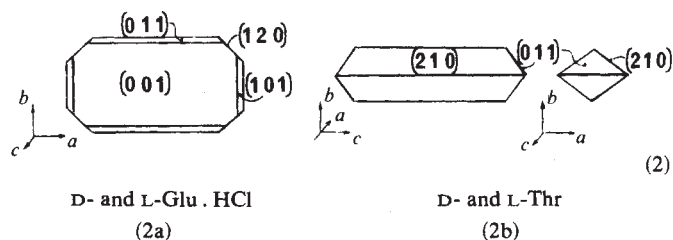
Table 1 Morphological separation of enantiomers of D, L-Asn crystallized in the presence of additives*

Additive (mg)	Morphology	Absolute configuration	[α] _D	Enantiomeric purity (%)†
L-Asp (10)	Unchanged	D	−28	91
	Changed	L	+31	100
D-Asp (20)	Unchanged	L	+28	92
	Changed	D	−23.6	78
L-Glu (20)	Unchanged	D	−28.6	94
	Changed	L	+29.4	97
D-Glu (50)	Unchanged	L	+27.6	91
	Changed	D	−23.5	84
L-Ser (25)	Unchanged	D	−29.7	98
	Changed	L	+28.5	94
L-Gln (50)	Unchanged	D	−29.7	98
	Changed	L	+28	92
L-Lys (25)	Unchanged	D	−24.4	80
	Changed	L	+26	86
L-His (50)	Unchanged	D	−18.5	61
	Changed	L	+28	92
L-Orn (25)	Unchanged	D	−27	89
	Changed	L	+26.5	87

* From aqueous solutions of concentration 300 mg per 3 cm³.

† Samples ranging from 70 to 80 mg in weight.

Figure 2 presents histograms of the measured areas for all the faces of typical crystals of pure L-Asn, L-Asn + L-Gln, L-Asn + L-Ser, L-Asn + L-Asp, and L-Asn + L-Orn, together with computer-drawn stereoscopic pictures of the crystals. Comparison of the histograms shows that, for the impurities L-Ser and L-Gln (Fig. 2B, C), the faces whose areas are relatively enhanced are {101} and, to a smaller extent, {111}, whereas the {011} and {012} faces are reduced in size. L-Glu, L-Orn, L-Lys and L-His induce similar morphological changes but, for the last three (Fig. 2E), the {111} faces are more developed than {101}. L-Asp, in contradistinction, causes L-Asn crystals to assume a completely different platelet-like morphology with predominant faces {010} (see Fig. 2D).



The influence of impurities on the two substrates (2a) and (2b) are described below.

D, L-Glu . HCl crystallizes in space group P2₁2₁2₁ (refs 22, 23) exhibiting a morphology depicted in scheme (2a). Crystallization experiments were performed by dissolving 1 g D, L-Glu . H₂O in 5M HCl (5 cm³) together with variable amounts of resolved L' impurity, filtering the hot solutions and cooling to room temperature (100% supersaturation).

Addition of small amounts (0.5 mg) of seed crystals of racemic Glu . HCl to the cooled supersaturated solution resulted in the initial separation of big crystals of D-Glu . HCl with a morphology as in Fig. 3a. In a second stage (after hours or days depending on the efficiency and concentration of the impurity)

there appeared crystalline powder composed of the L-enantiomer (Fig. 3f). On the basis of resolution power and morphological change the effective impurities may be divided into two groups, one consisting of the basic amino acids L-Lys . HCl, L-Orn . HCl and L-His . HCl, and the other of L-Cys . HCl, L-Thr and L-Ser. The former group, when used in high concentration (above 200 mg per 5 cm³ for His, 100 mg per 5 cm³ for Lys and Orn), strongly depresses the growth of L-Glu . HCl crystals which, however, eventually separate out in powder form (Fig. 3e). When Orn, Lys and His are added in concentrations as low as 10–25 mg per 15 cm³, morphological resolutions can still be performed successfully, the L enantiomer crystallizing in the form of thin {001} platelets (Fig. 3b–d). The second group of impurities, Cys . HCl, Thr, Ser, are effective to a lesser extent: the maximal morphological change consists only of a decrease of the thickness of the {001} crystal plates. HPLC experiments, performed as for Asn, showed preferential adsorption on L-Glu . HCl of L' impurity which again proved to be present throughout the bulk of the crystal at a uniform concentration (0.5), while D' is present only in the surface layers of the crystal.

D, L-Thr also crystallizes in space group P2₁2₁2₁ (ref. 24) with a morphology as shown in scheme (2b). The most effective tested impurities in the case of Thr are resolved Glu, Asp, Asn and Gln. The crystals obtained from 100% supersaturated filtered solutions (900 mg D, L-Thr in 5 cm³ pure water), in the presence of these impurities, appear in the form of bars coated with powder (Fig. 3b). As the crystals twin easily, morphological studies were best performed by separate crystallization of L-Thr in the presence of L' and D' impurities, performed in parallel in identical conditions. Photographs of crystals of pure L-Thr (Fig. 3g) and of L-Thr crystallized in the presence of increasing amounts of L-Glu (Fig. 3h–j) show gradual morphological modification. On the other hand the morphology of L-Thr is left unchanged by the presence of the same amounts of D-Glu. The impurities mainly affect the growth of the {210} faces of the bar-like crystals, turning these crystals first into thin needles and eventually into powder.

Discussion

Having characterized the morphological changes in terms of specific directions of crystal growth we shall now investigate whether we can obtain information about the situation of the occluded molecule in the substrate lattice, and the associated question of the correlation of the stereochemistry of the impurity molecule with its morphological influence.

We note that well-developed faces arise from strong attractive forces between molecules forming a layer parallel to the face. The tendency for growth in a certain direction depends on the attachment energy between the layer of molecules on the crystal face and the rest of the crystal²⁵. The habit changes indicate that introduction of the impurity perturbs the layer and the attachment energies for the various faces to different extents.

In all three systems Asn . H₂O, Glu . HCl and Thr, the effective impurity molecules have an arrangement of the carboxyl and amino groups directly attached to the asymmetric carbon identical to that of the host, but with different side chains. In crystals of all three substrates (Figs 4–6) the amino and carboxyl groups attached to the asymmetric carbon atom participate in several H-bonds. We expect that, in the 'doped' crystals, the H-bonding groups of the occluded impurities will occupy the same positions as do the corresponding groups of their host substrates, as they supply equivalent strong interactions. Thus the side chains of the impurities replace those of the substrates, but introduce contacts which differ from those of the host molecule. We shall account for the changes in crystal habit in terms of the effects of the contacts made by the impurity side chain at various crystal faces.

In the crystal structure L-Glu . HCl each side chain is directed approximately along the *c*-axis and makes H-bonds in this direction (Fig. 4). Thus it is clear why impurities such as L-Lys . HCl, L-Orn . HCl, L-His . HCl, L-Thr and L-Ser inhibit growth along the *c*-direction, decreasing the thickness of the {001} plates of the crystal.

We first examine the striking morphological modification brought about by L-Asp on L-Asn, where the essential structural change at the site of the impurity is the removal of one H-bonding amide H atom of Asn. Contrary to other additives, which are found in the bulk of the substrate crystal in amounts ranging from 0.5 to 1%, up to 12% of L-Asp has been found in single crystals of L-Asn, indicating formation of solid solution between additive and host. We therefore measured the cell dimensions of several pure and affected L-Asn crystals (four of each). The average cell dimensions of pure L-Asn are *a* = 5.582(2), *b* = 9.813(3), *c* = 11.791(4) Å, those of L-Asn + 10% L-Asp are *a* = 5.567(3), *b* = 9.835(5), *c* = 11.785(3) Å. These axial changes are compatible with the occluded Asp having its carboxy group in a *cis* rather than *trans* configuration. (There is much literature evidence in favour of the *cis* O–H bond. The *trans* O–H bond has not been found, with one exception²⁶, when O–H participates in an intramolecular O–H...O bond²⁷.) The shortening along *a* of 0.015 Å is in keeping with replacement of the N–H(*cis*)...O bond, of length 2.94 Å and directed with a

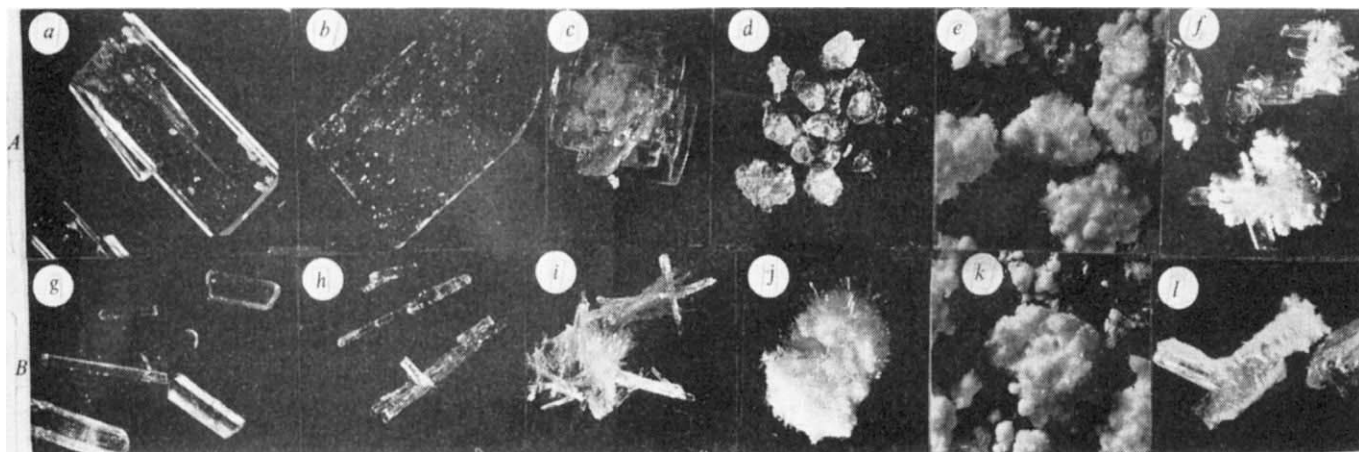


Fig. 3 A, Crystals of L-Glu . HCl grown in the presence of increasing amounts of impurity: a, none or D-Glu + L-Lys; b, + 2 mg cm⁻³ L-Lys; c, + 5 mg cm⁻³ L-Lys; d, + 10 mg cm⁻³ L-Lys; e, + 50 mg cm⁻³ L-Lys. f, Crystals of D, L-Glu . HCl grown in the presence of L-Lys. The plates are the D-enantiomer while the powder is the L-enantiomer. B, Crystals of L-Thr grown in the presence of different amounts of L-Glu; g, none or D-Thr + L-Glu; h, + 2.5 mg cm⁻³ L-Glu; i, + 7.5 mg cm⁻³ L-Glu; j, + 15 mg cm⁻³ L-Glu; k, + 30 mg cm⁻³ L-Glu. l, Crystals of D, L-Thr grown in the presence of L-Glu. The bars are the D-enantiomer while the powder is the L-enantiomer.

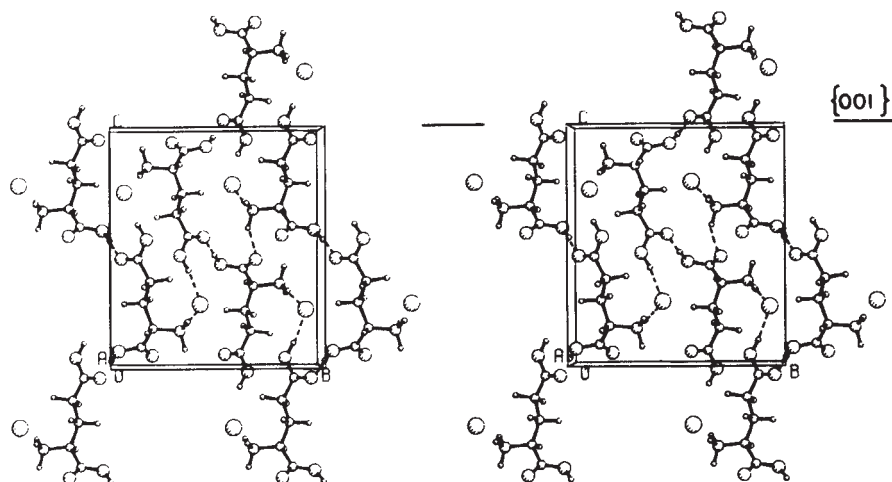


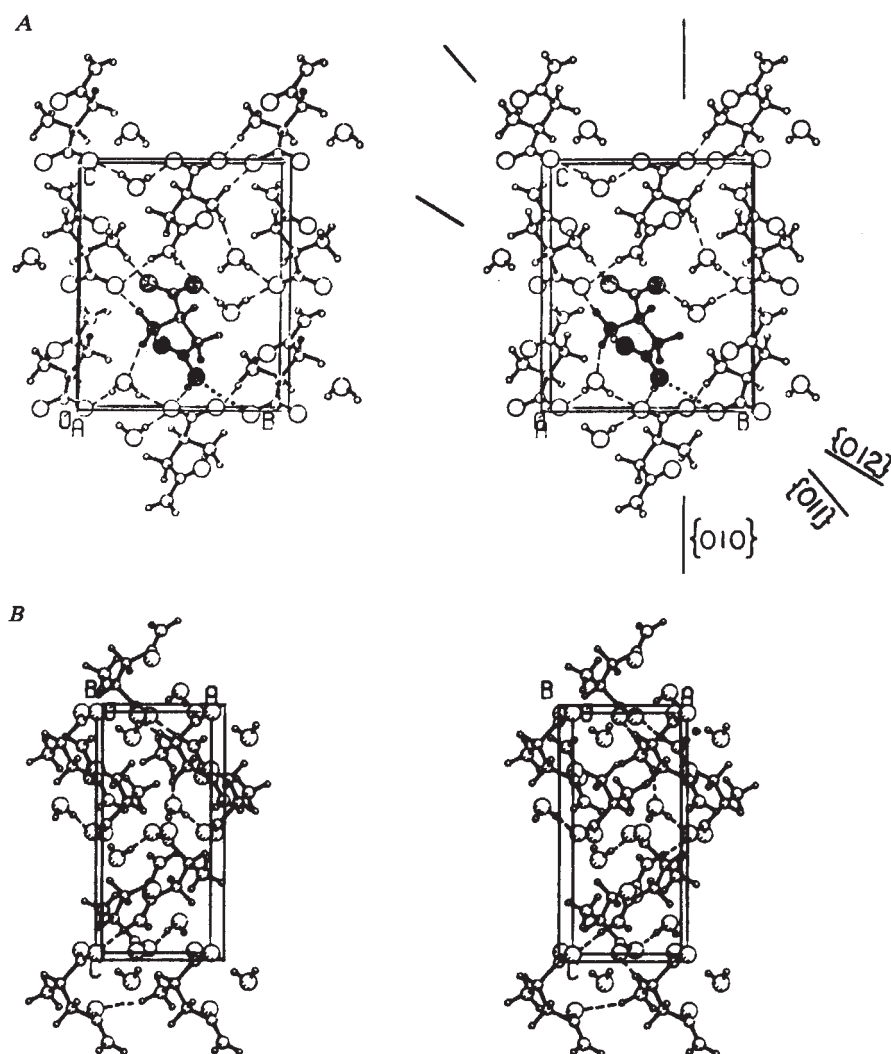
Fig. 4 Stereographic packing diagrams for L-glutamic acid . HCl as viewed along the a -axis.

large component along a (Fig. 5), by an OH(*cis*)...O bond which tends to be 2.6 Å in length. The increased length of the b axis of 0.022 Å is in keeping with a replacement of the N-H(*trans*)...O bond, of length 3.05 Å and directed mainly along b (Fig. 5A), by an O...O contact which may be as long as 3.5 Å; this O...O interaction is repulsive because the lone pair electrons of these two atoms are directed towards each other²⁶⁻²⁹. Indeed these two new interactions introduced by the impurity, namely the attractive O-H...O and the repulsive O...O ones, may explain the formation of {010} plates of the affected crystal. The {010} layer energy is enhanced by the former, while the {010} attach-

ment energy is severely reduced by the repulsive O...O interaction, thus developing large {010} faces.

The impurities L-Gln, L-Glu, L-Orn, L-Lys, L-His and L-Ser on L-Asn all have the same general effect: the preferred crystal growth along the a direction is replaced by a tendency for growth along b , and concomitantly with a reduction in area of the {011} faces. We may rationalize these morphological changes as follows. First, the amide N-H(*cis*)...O (carboxy) and amino N-H...O (amide) bonds, which are directed mainly along the a -axis (see Fig. 5B) would be absent at the site of impurity, thus inhibiting growth along a . Furthermore the Asn molecules

Fig. 5 Stereographic packing diagrams for L-asparagine . H₂O. A, view along the a -axis. Here one asparagine molecule is replaced by an aspartic acid molecule (filled circles). Some of the crystal faces affected by the impurities are indicated. B, view along the b -axis.



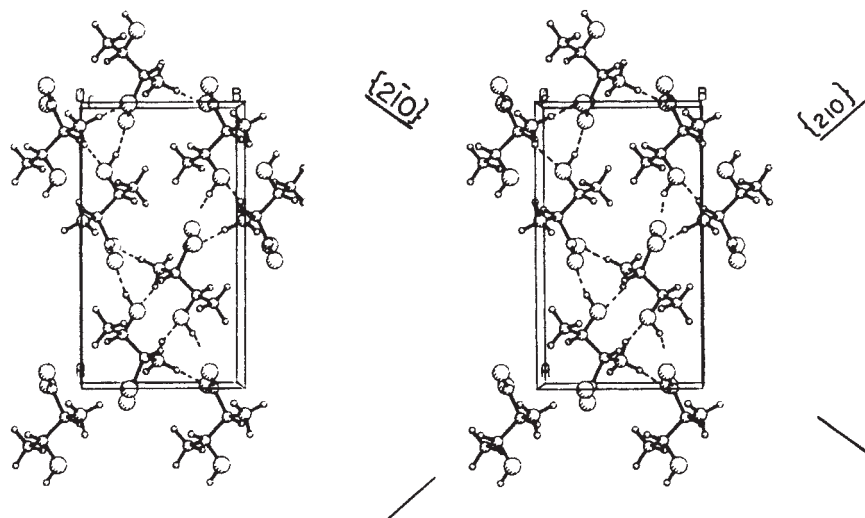


Fig. 6 Stereographic packing diagram for L-threonine as seen along the *c*-axis. The directions parallel to the affected faces are indicated.

related by the *a* translation form the highest number of contacts per pair of molecules, and thus growth along this direction should be the most affected. The relative increase of growth along *b* for affected crystals may be appreciated when we note that only one amide N-H...O bond directed almost along *b* would be absent at the site of the impurity.

The rupture of the OH...O bond of the side chain hydroxyl of the substrate Thr at the site of the impurity would certainly inhibit growth of the {210} faces perpendicular to the needle *c*-axis, as the O-H...O bonds are directed almost parallel or perpendicular to these faces, thus lowering both their layer and attachment energies (Fig. 6). However, the growth along the needle *c*-axis should not be dampened. Thus the observed effect of impurities, including thinner needles and eventually powder, is compatible with such a model.

Conclusions

On the basis of all our data, we suggest a mechanism involving two steps:

(1) Binding: the strong interactions between the amino acid group $\text{NH}_3^+ - \text{CHCOO}^-$ of the substrate and the molecules at the growing site lead to binding of the impurity molecule at the surface of the crystal, in the same way as a substrate molecule is bound. A high degree of stereospecificity is needed to fit the chiral additive to the structured surface, and this explains the L'/D' selectivity of the effect.

(2) Inhibition: once the impurity has been adsorbed at the surface, it disturbs the regular deposition of the next layer of substrate molecules in the specific direction along which the

modified side chain group emerges. The obstacle is eventually removed by overgrowth resulting in the inclusion of the foreign material in the bulk of the crystal. This mechanism would rationalize the different inhibition power of the various impurities, in terms of the hydrogen bonding and acid-base properties of the side chains of these molecules (for example, the strong effect of L-Lys, L-His and L-Orn on L-Glu · HCl).

It is instructive to consider the implication of this mechanism for crystals containing a polar *b* axis. Here the faces (*hkl*) and (*h $\bar{k}l$*) have different structures³⁰⁻³³. Thus their growths can be affected differentially by an appropriate 'tailor-made' impurity. The specific morphological changes induced would then allow us to determine the absolute configuration of the substrate akin to the Bijvoet method. Studies in these directions on the systems L-Lys · HCl, and (L) cinnamoyl alanine, have been successful and are reported in the following paper³⁴.

Finally, apart from providing us with a method for controlled modification of crystal morphology, this work allows a deeper understanding of the role of the tailor-made impurity thus strengthening the empirical approach for assignment of relative configuration we proposed previously¹⁰, it also enables us to select better additives for efficient kinetic large scale resolution of conglomerates, and for growing single crystals of interest for material science and crystallographic measurements.

Parallel calculations of intermolecular interactions presently in progress, using existing techniques³⁵, have already provided a quantitative basis for the mechanism proposed above, for simple systems such as benzamide-benzoic acid (Z.B.-Y., L.A., M. Idelson, M.L. and L.L. work in preparation).

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Absolute configuration of chiral polar crystals

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A method is described for direct determination of absolute configuration of chiral polar crystals, based on changes in crystal habit induced by tailor-made impurities. The method embodies the assignment of the direction of the chiral substrate molecule with respect to the crystal polar axis based on differences in the hemihedral faces of the substrate crystals as grown from solution in the presence and absence of impurities. The molecular packing requirements and the choice of impurities for application of this method in lysine and trans-cinnamoyl alanine are outlined.

THE configuration of chiral molecules had been defined only relatively, until 1951 when Bijvoet applied the method of anomalous dispersion of X rays to crystals of ammonium rubidium tartrate, thus placing stereochemistry on an absolute scale¹. In 1973 it was shown that two other, independent methods, noble gas ion reflection mass-spectrometry² and circular dichroism³⁻⁵, give chirality assignments which are in accord with the assignments according to Bijvoet (see review in ref. 6).

The assignment of the absolute configuration of chiral molecules in chiral crystals which develop hemihedral faces has long challenged the chemical crystallographer. In principle any method which establishes the orientation of a given functional group of the molecule with respect to the crystal axes or to a hemihedral face bounding the crystal, fixes the absolute configuration of the molecule. Several attempts have been made to deduce absolute configuration from crystal habit. Waser⁷ tried to establish the absolute configuration of D-tartaric acid by correlating the relative rates of growth of the hemihedral (*hkl*) and ($\bar{h}\bar{k}\bar{l}$) crystal faces with the ease of attachment of the 'free' molecule at either face. However, Bijvoet⁶ later showed Waser's conclusions to be incorrect. Indeed, Turner and Lonsdale⁸ had earlier criticized Waser's reasoning, arguing that, within the limits of his assumptions, the ease of approach at either face is identical. In 1949 Wells⁹ examined the differences in the habit and growth directions of the polar crystals of resorcinol grown from various solvents. He interpreted the solvent effects as being due to the different interactions of the solvent with the different exposed faces of the crystal, which have polar OH groups at one end and hydrophobic benzene groups on the other. Booth and Bukley¹⁰ proposed that the same kind of interactions may be responsible for the growth behaviour of ethylenediamine tartrate (monohydrate) crystals, pure or in the presence of boric acid. Paul, Curtin and co-workers^{11,12} recently correlated the direction of the unique polar axis of *p*-bromobenzoic acid anhydride crystal with that of the preferential attack of ammonia gas on appropriate faces of single crystals of this compound.

Our recent studies on morphological changes in organic crystals as induced by 'tailor-made' impurities¹³ suggested a new direct method for the assignment of absolute configuration of chiral crystals and molecules. This method embodies the establishment of the orientation of the chiral molecule with respect to the crystal polar axes.

Crystal morphology change and nature of added impurity

Kinetic resolution of a large variety of racemic conglomerates in the presence of tailor-made impurities revealed, in many systems, stereoselective adsorption of the impurity at the surface of one enantiomorph, followed by drastic changes in its crystal habit, while the other enantiomorph remains unchanged¹⁴. Through these habit changes, a stereochemical correlation has been established between the molecular structure of the

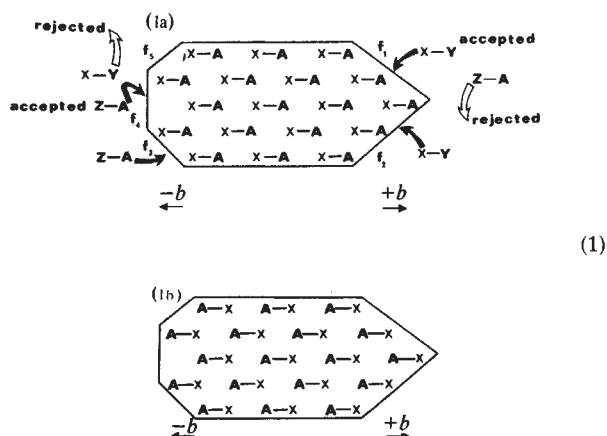
impurity, the crystal structure of the substrate, and the affected growth directions.

A detailed study of such morphological changes induced in the crystals of L-asparagine.H₂O, L-threonine and L-glutamic acid.HCl, by other L-amino acids used as additives, led to the proposal of a two-step mechanism of binding and inhibition¹³. By virtue of strong attractive interactions (H-bonds, ionic) the unmodified (with respect to substrate molecules) amino acid group of the impurity binds stereospecifically at a site suited to the amino acid group of the substrate molecule on the surface of the host crystal. However, the modified chain of the occluded impurity perturbs the regular deposition of the oncoming layers of the crystal in specific directions, as defined by the hydrogen bonding pattern of the side chain of the substrate. The rate of growth in these directions is thus decreased, leading to changes in crystal morphology. These results, which have been extended to other hydrogen bonded systems (Z.B.-Y., L.A., M.L. and L.L., work in preparation), suggested that crystal habit can be modified in a controlled way by use of appropriate crystal growth inhibitors.

We have applied this concept to polar crystals, racemates and meso compounds to design a new direct method for the assignment of absolute configuration of chiral molecules. Although here we consider only polar crystals. We shall first present a topological model which outlines the requirements for assignment of absolute configuration, and then describe its successful application to Lys.HCl.2H₂O and trans-cinnamoyl-alanine.

Modelling impurity effects on polar crystal growth

We consider a chiral molecule (or a non-chiral molecule adopting a chiral conformation), represented schematically as X-A, packing in a polar crystal whose unique polar axis is parallel to the X-A molecular axis. These requirements can be fulfilled in all chiral polar space groups, among which are P1, P2₁, C2, and



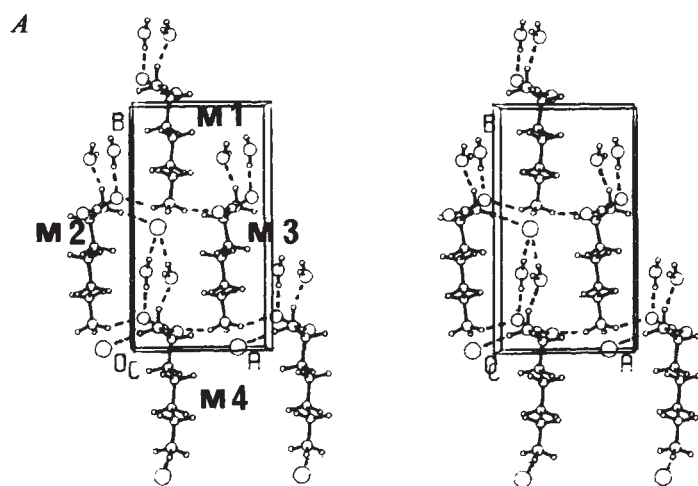
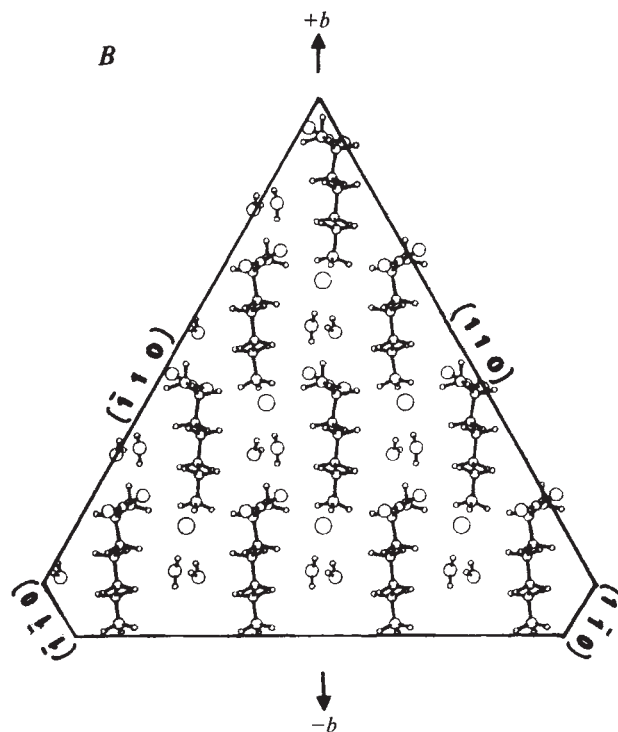


Fig. 1 Packing arrangement of the crystal structure of L-lysine $\cdot$ HCl $\cdot$ 2H₂O viewed along the c -axis. **A**, stereographic view; **B**, packing arrangement as delineated by the observed $hk0$ crystal faces.



P3₁. In principle the approach may be extended to any chiral crystal (such as P222, P2₁2₁2₁), in which the molecule X-A is oriented in a general polar direction.

The crystal model is depicted in scheme (1) for a structure appearing in the common polar space group P2₁. This crystal is delineated by faces f_1, f_2 in the $+b$ -direction and f_3, f_4, f_5 in the $-b$ -direction. By virtue of the crystal (point) symmetry the faces within each pair (f_1, f_2) and (f_3, f_4) are related by 2-fold symmetry. The polar nature of the crystal precludes a symmetry relationship between the $+b$ and $-b$ directions. Thus faces within the pairs (f_1, f_5) and (f_2, f_3) cannot be related by symmetry. Consider that in this crystal only A groups emerge at faces f_1 and f_2 whereas X groups emerge at faces f_3, f_4, f_5 . The essence of the problem is that conventional X-ray diffraction does not provide information as to whether the molecule X-A points in the $+b$ or in the $-b$ direction. In other words, it does not allow one to distinguish between the real crystal scheme (1a) and the hypothetical enantiomeric structure in which the orientation of X-A with respect to the b -axis is opposite scheme (1b).

Applying the two-stage mechanism of adsorption-inhibition, described above, appropriate impurities X-Y or Z-A should influence asymmetrically the growth rate of the crystal along the polar b -axis, thus allowing one to determine whether X-A is directed along the $+b$ - or $-b$ -direction, through selective morphological changes. An impurity X-Y will bind to faces f_1 and f_2 , but not to f_3, f_4, f_5 , and once bound, will hinder growth along the $+b$ and possibly other directions, but not along $-b$ in the real crystal of scheme (1) (the opposite would happen in the crystal of scheme (1b)). We would then predict changes in crystal habit involving either an increase of the areas of faces f_1 and f_2 relative to those of other faces of the crystal, or the development of appropriate, additional new faces. The same logic applies to an impurity Z-A which we predict would affect growth of faces f_3, f_4, f_5 . The morphological differences between crystals grown in the presence and in the absence of the impurity would then indicate the direction of the modified group in the given crystal, thus establishing the direction of the substrate molecule X-A with respect to the polar axis. Consequently the absolute configuration of the crystal and of the chiral molecular constituents can be derived. Note that the additive need not be chiral and, even if it is, the assignment of absolute configuration of the crystal does not require a knowledge of the absolute configuration of the additive.

Results

(L)Lys.HCl crystallizes from water as a dihydrate in a monoclinic structure¹⁵⁻¹⁷ of space group P2₁ with $Z = 2$ and $a = 7.49$,

$b = 13.32$, $c = 5.88$ Å, $\beta = 97.50^\circ$. The packing arrangement viewed along the c -axis is shown in Fig. 1. The Lys molecules are oriented with respect to the b -axis so that the ϵ -NH₃⁺ group is directed along $-b$, and the amino acid function (NH₃⁺C(CO₂⁻)) along $+b$. Crystals of (L)Lys.HCl.2H₂O grown from water (900 mg cm⁻³, cooling in the presence of seeds, 0.5 mg), appear mainly as prisms with an almost triangular section, with the b -axis bisecting the triangle and c directed along the long axis of the prisms (Figs 1B, 2A). The orientations of the molecules relative to the observed $hk0$ crystal faces are shown in Fig. 1B.

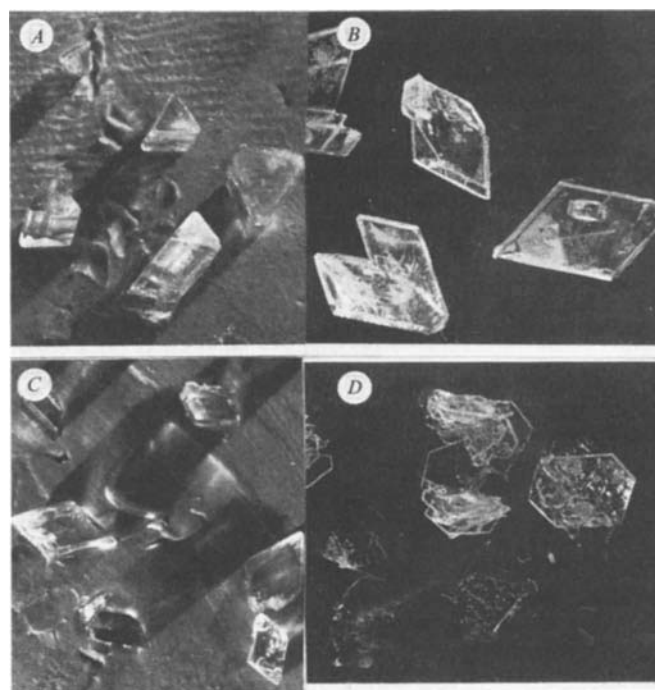


Fig. 2 Crystals of L-lysine.HCl.2H₂O grown in the absence and presence of impurities: **A**, pure; **B**, +additive L-Orn; **C**, +L-NorVal; **D**, +L-MeLys.

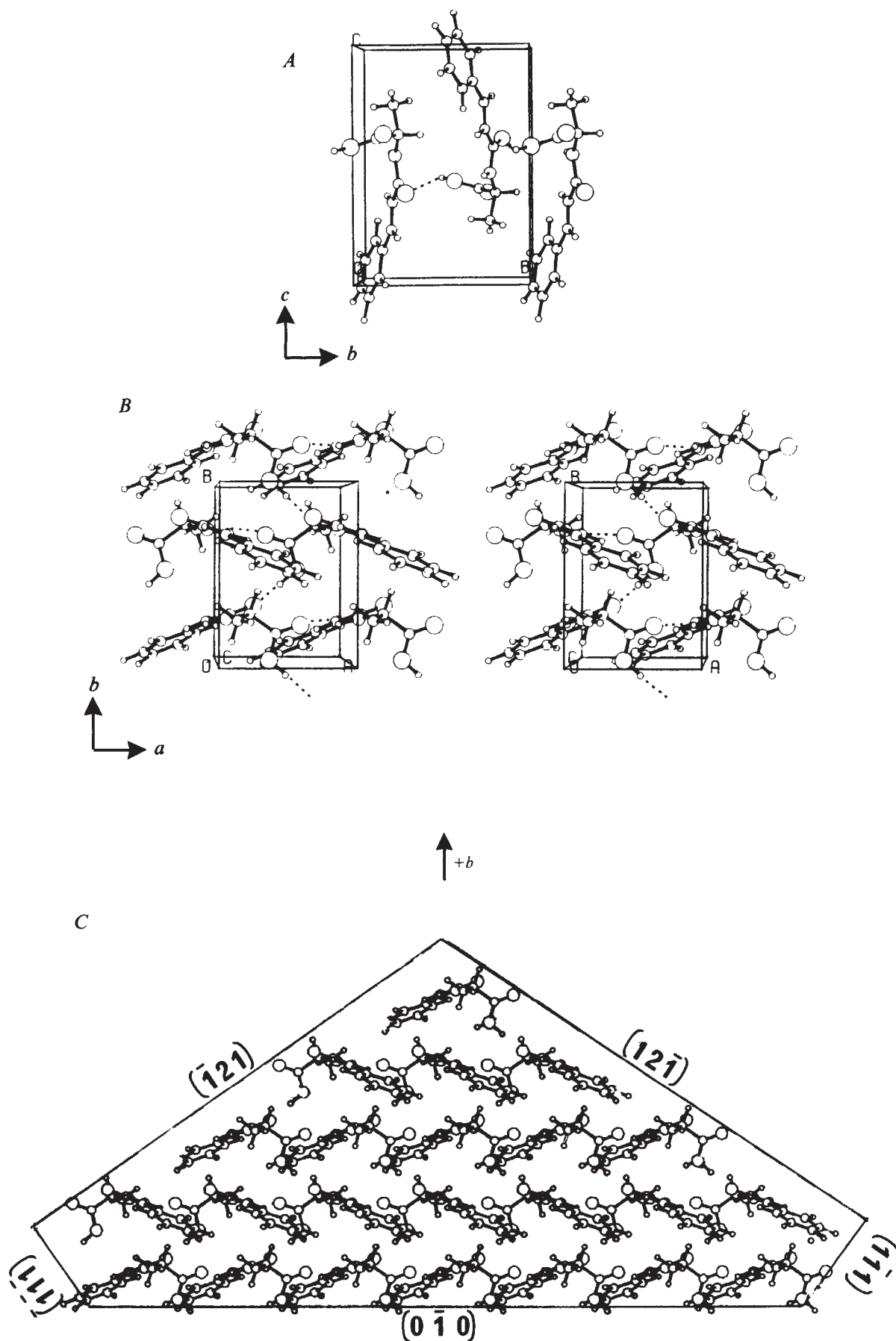


Fig. 3 Packing arrangement of *trans*-cinnamoyl-L-alanine: *A*, viewed along the *a*-axis; *B*, viewed along the *c*-axis; *C*, viewed along the *c*-axis as delineated by the observed (*h**k*0) crystal faces.

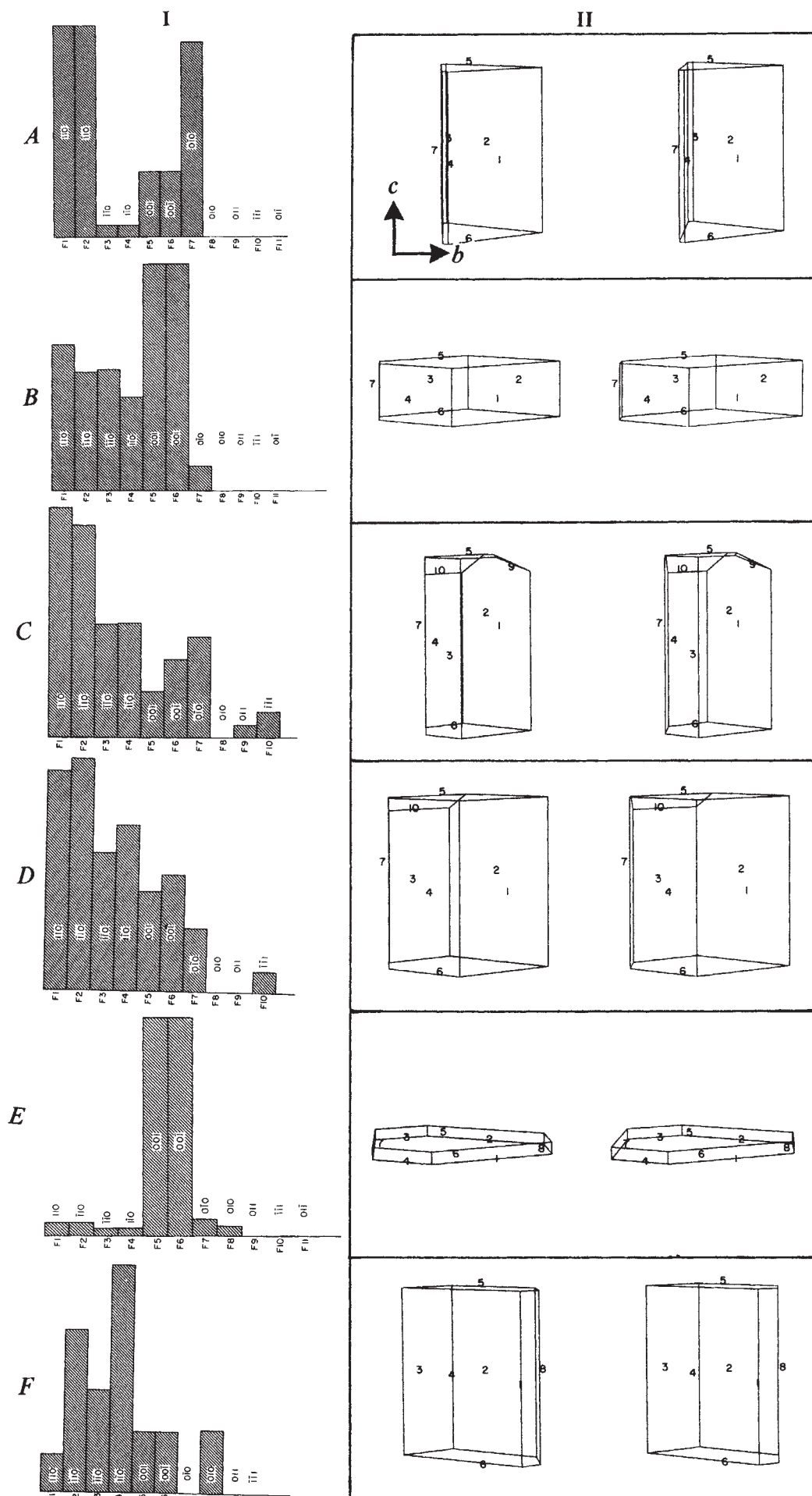
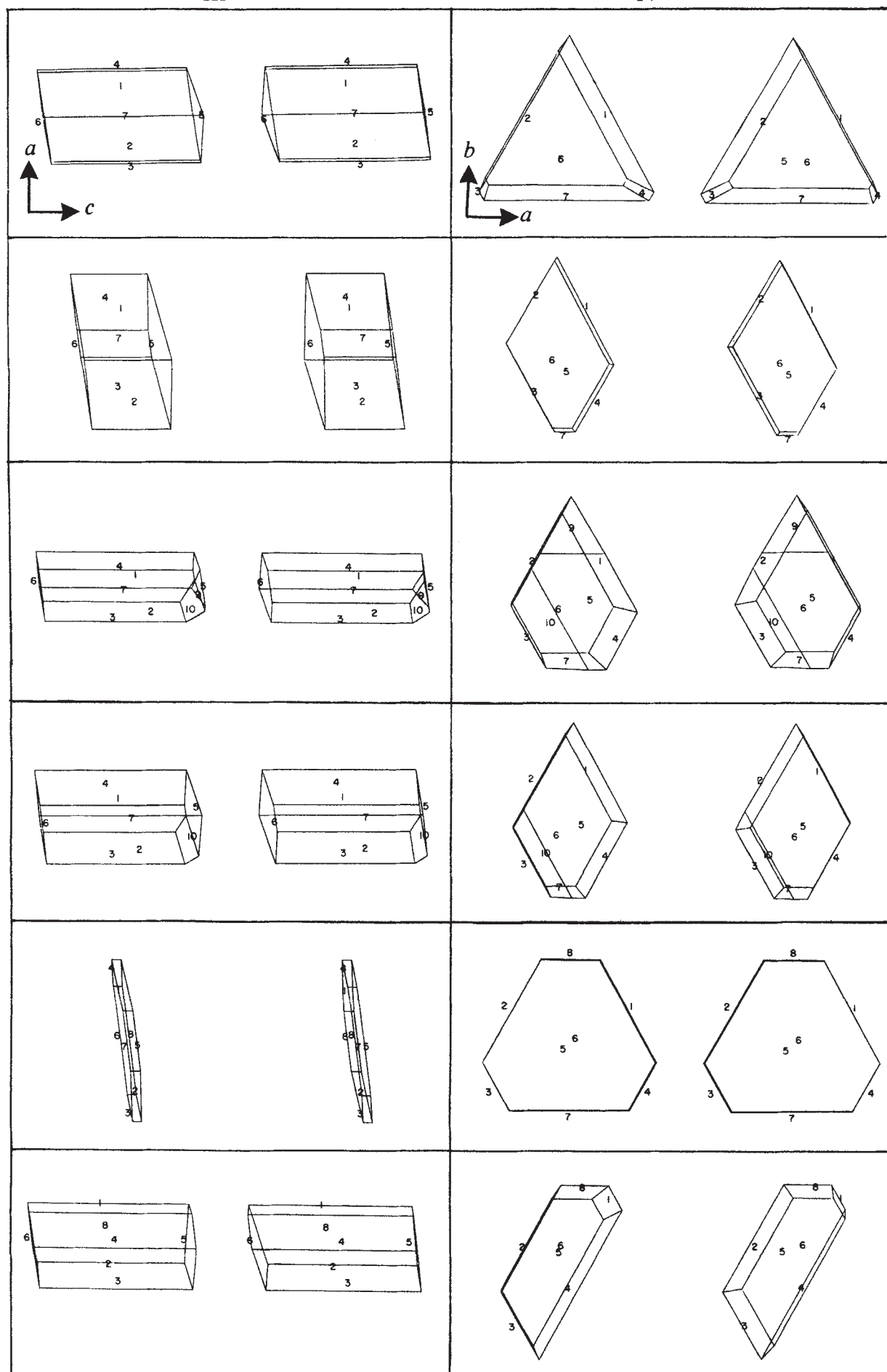


Fig. 4 A comparison between crystals of L-lysine.HCl.2H₂O grown in the absence and presence of additives: *A*, pure; *B*, +L-Orn; *C*, +L-NorVal; *D*, +L-NorLeu; *E*, +L-MeLys; *F*, +ε-aminocaproic acid. **I**, Histograms describing the areas of the faces. **II**, **III** and **IV**, computer-drawn stereographic pictures of the crystals viewed along axes *a*, *b* and *c* respectively.

III

IV



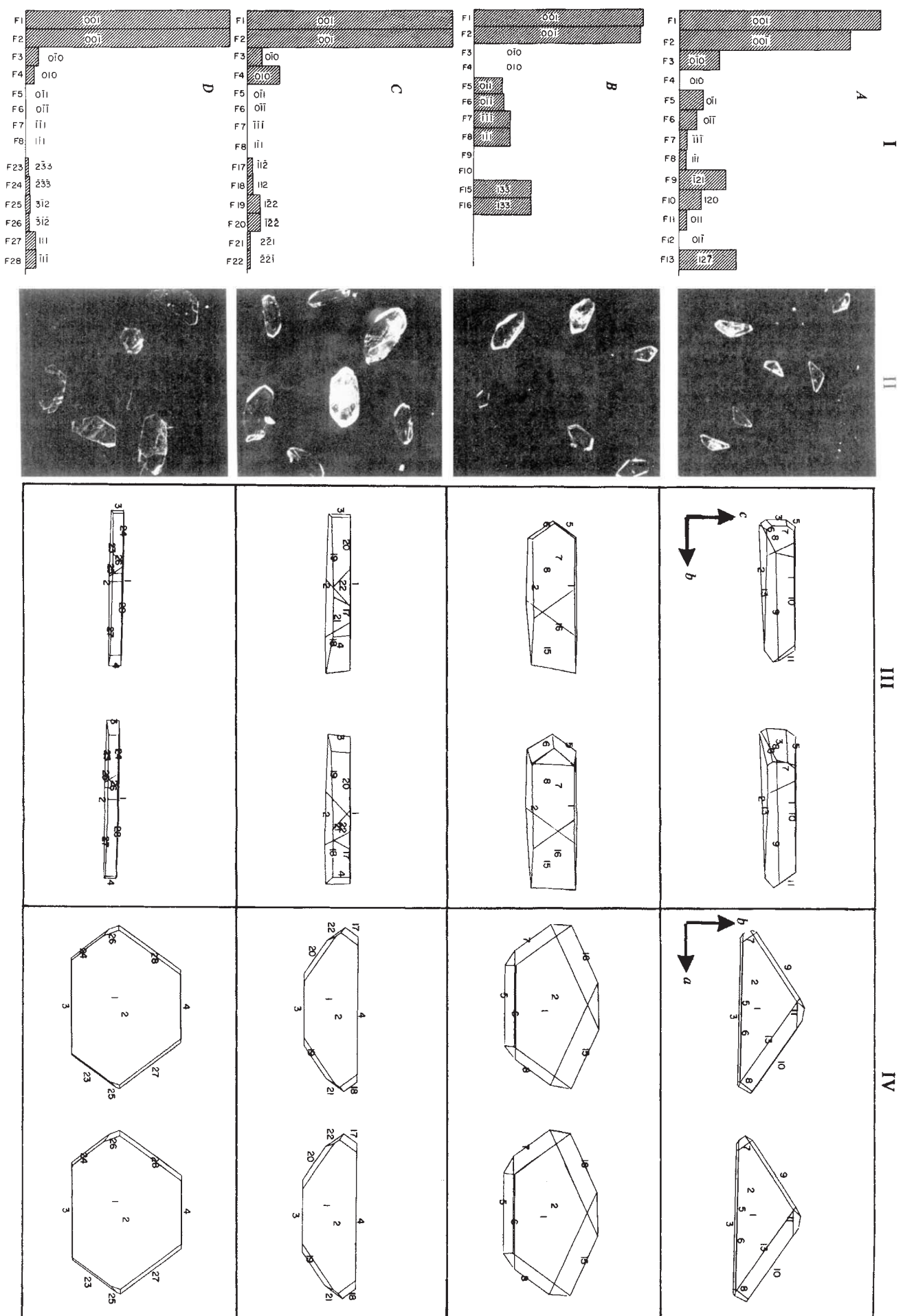


Fig. 5 A comparison between crystals of cinnamoyl L-alanine grown in the absence and presence of additives: A, pure; B, + 3% methyl ester; C, + *trans*-cinnamoyl (D)-alanine; D, + *trans*-cinnamoyl D-serine. I, Histograms describing the areas of the faces. II, Photographs of crystals. III and IV, Computer-drawn stereographic pictures of the crystals viewed along axes *a* and *c* respectively.

Bearing in mind the above conclusions, the crystal structure suggested that an impurity with modified carboxyl or α -amino groups will disturb growth along $+b$ but not along $-b$, whereas modifications in the side chain will, by contrast, disturb growth along $-b$, but not $+b$. For both cases we may expect some modification in the *a* and *c* directions. (1) L-methyl lysinate (MeLys) and ϵ -aminocaproic acid (which is non-chiral) and (2) L-ornithine (Orn), L-norleucine (NorLeu), and L-norvaline (NorVal) were selected as additives representative of the two cases. Crystals of L-Lys.HCl.2H₂O, were grown from water in the presence of each impurity separately. The concentrations of the additives in solution were: L-Orn.HCl: 200 mg cm⁻³; L-NorLeu: 10 mg cm⁻³; L-NorVal: 10 mg cm⁻³; L-MeLys.HCl: 50 mg cm⁻³; ϵ -aminocaproic acid: 200 mg cm⁻³. The amounts of additives used in solution were chosen on the basis of the respective solubilities in H₂O, and are always much larger than the amounts found in the bulk of the substrate crystals after crystallization.

The crystals obtained were separated by decantation, dried and examined (Fig. 2B–D). The faces were characterized and the dimensions measured as described previously¹³. The computer-drawn pictures of pure and affected crystals are shown in Fig. 4 together with the histograms of the face areas. The additives L-Orn, L-NorLeu, and L-NorVal inhibit growth along the $-b$ -direction, as indicated by the pronounced increase in the areas of the symmetry-related ($\bar{1}\bar{1}0$) and ($\bar{1}\bar{1}0$) faces (see Figs 2B, C and 4B, C, D). This change in crystal habit is primarily a consequence of rupture at the impurity site of the two (ϵ -amino)N–H...O(carboxyl) H-bonds linking molecule M(1) to M(2) and M(3), related to it through 2₁-axes (see Fig. 1A). The H-bonded system (ϵ -amino)N–H...Cl⁻ along the $-b$ -direction would also be hindered.

The impurity L-MeLys affects growth along the $+b$ -direction inducing formation of the (010) face. Although we could not pinpoint the precise orientation of the O–CH₃ group of the impurity, there can be little doubt that this group would disrupt the H-bonded network along the $+b$ -direction because the substrate CO₂⁻ group participates in several H-bonds along $+b$, from molecule M(4) to M(1) and M(3) and to two water molecules (Fig. 1). MeLys also affects growth along the *c*-axis, turning the crystal into (001) plates (Figs 2D and 4E). This change is not surprising in view of a possible rupture of the (ϵ -amino)N–H...O(carboxyl) bond interlinking lysine molecules along the *c*-axis and of the steric hindrance induced by the methyl group of MeLys along the short *c*-axis. ϵ -Aminocaproic acid induces formation of the (010) crystal face and thus has a similar effect to MeLys, which may be explained on analogous lines. However, unlike MeLys, this impurity does not affect growth along the *c*-axis, presumably because no steric hindrance is induced along this direction.

Trans-cinnamoyl-L-alanine

Trans-cinnamoyl-L-alanine crystallizes from CHCl₃, MeOH or EtOH in polar crystals of space group P2₁, with *a* = 6.1, *b* = 8.2, *c* = 11.3 Å, β = 90.6°. In the crystal^{18,19} (Fig. 3) the molecules are oriented such that the carboxyl O–H participates in an O–H...O(amide) bond whose vector has a major component along the $-b$ -direction, and the C(chiral)–H bond is almost parallel to $+b$ (Fig. 3A). Pure *trans*-cinnamoyl-L-alanine crystals, grown from CHCl₃ by slow evaporation at room temperature, appear as thin pentagonal (001) platelets, with the symmetry axis *b* bisecting the pentagon perpendicular to its base (Figs 3C, 5A). Figure 3C shows the orientation of the molecule with reference to crystal faces in the pure crystal. Following the above analysis, the methyl ester of the acid should decrease the

growth rate in the $-b$ -direction. By the same reasoning growth should be inhibited along the opposite direction, $+b$, using *trans*-cinnamoyl-D-alanine or *trans*-cinnamoyl-D-serine as the impurity: binding at the surface of the substrate crystals should be possible for such impurities by virtue of strong H-bonds, but once bound their C(chiral)–CH₃ [or C(chiral)–CH₂OH] group would replace the C(chiral)–H group of the substrate thus hindering growth along the $+b$ -direction. Such a conformation of the impurity molecule would not incur intramolecular steric contacts. Indeed the ease of such an effective interchange of the CH₃ and H substituents at the chiral C atom is demonstrated in the crystal structure of *o*-chlorocinnamoyl-L-alanine^{18,19}.

Crystals were grown from CHCl₃ in the presence of increasing amounts (1, 3, 5 and 10% by weight) of these three impurities, in the same conditions as for the pure compounds. The morphologies of these crystals are presented (Fig. 5). The most pronounced change induced by the methyl ester of *trans*-cinnamoyl-L-alanine is the large increase in the areas of the symmetry-related ($\bar{1}\bar{1}1$) and ($\bar{1}\bar{1}1$) faces. Other changes are the formation of the (0 $\bar{1}1$) and (0 $\bar{1}\bar{1}$) faces instead of (0 $\bar{1}0$) and replacement of the steep ($\bar{1}21$) and (120) faces by the shallower (133) and ($\bar{1}33$) faces. The relative increase in areas of these faces is, by and large, proportional to the amount of impurity in solution (1–5%).

We account for the large increase in the area of the ($\bar{1}\bar{1}1$) face in terms of the observation that the (carboxyl)O–H...O(amide) H-bond emerges from this face almost perpendicular to it, making an angle of 16° with its normal. Thus the replacement of the substrate group by O–CH₃ at the site of the impurity precludes formation of the O–H...O H-bond and hinders the natural growth perpendicular to the ($\bar{1}\bar{1}1$) face, with a subsequent increase in its area.

The impurity *trans*-cinnamoyl-D-alanine induces formation of the (010) face as shown in Fig. 5C. This morphological change reflects an inhibition of growth along the $+b$ -direction, as predicted above. A similar morphological change is induced by the additive *trans*-cinnamoyl-D-serine (Fig. 5D). In the case of *trans*-cinnamoyl-D-alanine the effect is so pronounced that the (010) face, absent in the pure crystal, is much more developed than the naturally developed (010) face.

Discussion

The experimental findings on lysine and cinnamoyl alanine indicate that it is possible through modification of crystal morphology by tailor-made impurities in appropriate systems, to establish the absolute configuration of the substrate molecule. The present approach differs radically from that of Waser⁷, who tried to ascertain absolute configuration on the basis of the morphology of the pure crystal by comparing the relative ease of attachment of molecules to hemihedral faces $\{hkl\}$ and $\{\bar{h}\bar{k}\bar{l}\}$. The latter approach is full of pitfalls, because the sole difference between the energy of attachment²⁰ to the two faces arises from solvent effects and differences in polarizability and conformation between the crystal-bound molecule at the surface and the to-be-attached 'free' molecule. Effects of these kinds are difficult to quantify. These uncertainties do not affect our approach because the change in crystal habit originates essentially from the difference in the ease of approach of the to-be-attached molecule to the same face in the poisoned and unpoisoned crystals. Moreover, the reliability of the method is increased by observing a progressive change in crystal habit as a result of increasing concentration of impurity in solution, and by a cross-check of the assignment by using various impurities, appropriately designed, so that they will affect stereoselectively either polar growth direction.

The method is not limited to chiral crystals, but is applicable also to racemic or *meso* compounds, with certain desired

packing features, for assignment of the absolute configuration of the chiral additive. This extension is based on the fact that, in contradistinction to chiral crystals, in racemic compounds comprising chiral R and S moieties, one can specify unambiguously the chirality of the moiety which emerges from a given crystal face. Crystallization of such a racemic crystal poisoned with chiral impurity may affect enantioselectively one of the pair of enantiotopic crystal faces. From the change in crystal habit it may be possible to fix the absolute configuration of the additive. An analysis of the packing requirements and application of this method to serine-threonine will be presented elsewhere²¹.

The rules of morphological changes, as outlined here, also

apply to solid solutions, with the advantage that one may there be able to locate precisely the atomic positions of the occluded guest additive by X-ray diffraction. This will eventually allow us to study these phenomena on a quantitative basis, leading hopefully to a better understanding of the correlation between molecular structure and crystal morphology.

We thank Professor Duilio Arigoni for his prompting (since 1969) that there must be a simple way to extract absolute configuration of chiral molecules from morphology, also Professor M. D. Cohen for discussions and Sarah Ariel for the crystal structure coordinates of cinnamoyl-L-alanine, and the US/Israel Binational Science Foundation, Jerusalem, and the Volkswagen Stiftung Foundation for financial support.

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A monoclonal antibody defining antigenic determinants on subpopulations of mammalian neurones and *Trypanosoma cruzi* parasites

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*An IgM λ class monoclonal antibody raised against membranes from rat dorsal root ganglia defines a novel antigenic determinant expressed by subpopulations of mammalian central and peripheral neurones. In the presence of complement the antibody is cytotoxic to mammalian neurones in vitro. The same antibody labels *Trypanosoma cruzi*, the protozoan responsible for Chagas' disease. Classes of mammalian neurones and cardiac muscle that are labelled by the antibody are known to degenerate in Chagas' disease. The common neuronal and trypanosomal antigens recognized by the antibody may therefore be important in pathogenic events underlying this disorder.*

CHAGAS' DISEASE results from infection with the protozoan *Trypanosoma cruzi*, affects an estimated 12 million people in Latin America¹ and is characterized by extensive neuronal degeneration. In the peripheral nervous system, there is a massive degeneration of sympathetic², parasympathetic³ and enteric^{4,5} neurones. Lesions of Purkinje neurones in the cerebellar cortex have been reported^{5,6} and there is also significant degeneration of cardiac muscle in both the acute and chronic stages of the disease⁷.

Although neuropathy does not result from direct parasite invasion, the precise mechanism underlying neuronal degeneration is still unclear. It has been suggested that Chagas' disease may have an immunological basis, consistent with the destruction of cardiac and neuronal tissue by an autoimmune mechanism^{8,9}. One explanation for the origin of this autoreactivity is that the host and parasite share common antigenic determinants, such that the development of anti-parasite immunity allows cross-reactivity with self components.

Previous studies have demonstrated that sera from patients with *T. cruzi* infection contain antibodies that recognize mammalian vascular endothelial cells¹⁰, components of peripheral nerve, possibly Schwann cells¹¹, the plasma membrane of striated muscle and endothelial cells¹² and certain peripheral and central neurones¹³. However, in these studies the nature of the antigenic stimulus is unknown.

We report here that a monoclonal antibody, generated against mammalian dorsal root ganglia, exhibits a unique specificity for subpopulations of central and peripheral neurones and also recognizes an antigenic determinant on the protozoan *T. cruzi*. Strikingly, all classes of neurones we have examined that are known to undergo degeneration in Chagas' disease express this antigenic determinant. In addition, cardiac muscle is stained by the antibody whereas skeletal muscle, which is unaffected in Chagas' disease, is not.

Production and characterization of antibodies

With the original aim of generating antibodies that might provide markers for subpopulations of primary sensory neurones, dorsal root ganglia were dissected from 7-day-old CD rats (Charles River Breeding Laboratories). Ganglia pooled from

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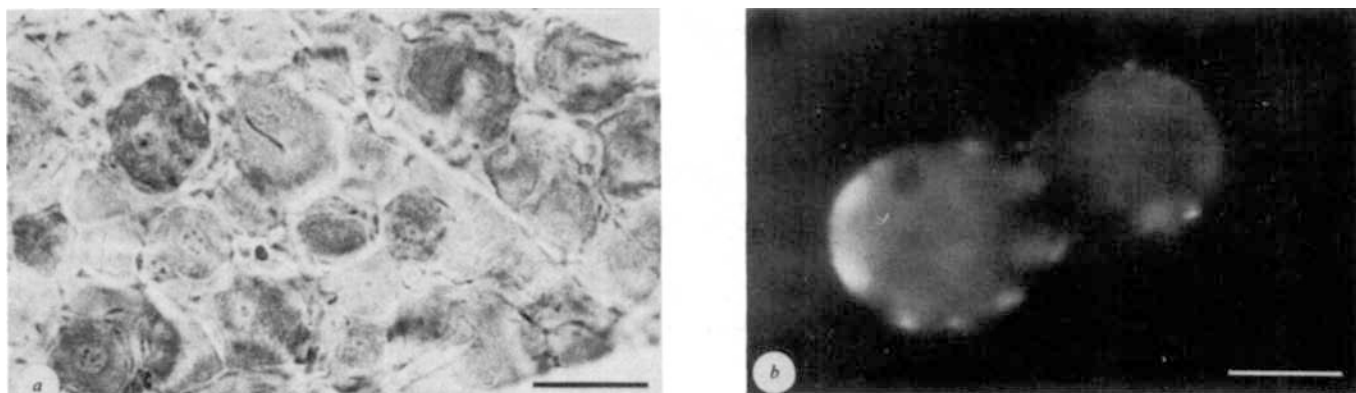


Fig. 1 Immunocytochemical labelling by CE5 of rat dorsal root ganglion neurones in fixed sections and dissociated cell culture. For staining of fixed sections, rats were anaesthetized with ether and perfused via the aorta with 500 ml of 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) at room temperature. The brain, spinal cord and dorsal root ganglia were removed and placed overnight at 4°C in 0.1 M phosphate buffer containing 30% w/v sucrose. 30- μ m sections were cut with a freezing microtome and incubated in CE5 hybridoma supernatant diluted 1:1 with PBS (pH 7.4) for 18–24 h at 4°C. Sections were then washed three times with PBS and incubated for 20 min with either horseradish peroxidase- or FITC-conjugated rabbit anti-mouse IgG (Litton Bionetics) diluted 1:25 with PBS. Sections processed for fluorescence were then washed three times in PBS mounted on glass slides, and viewed on a Leitz Ortholux II microscope equipped with epifluorescence illumination. Photographs were taken with 2-min exposures using 400 ASA film. For peroxidase immunocytochemistry, sections were washed three times in 0.05 M Tris-HCl (pH 7.6) after removal of second antibody and incubated with 0.05% diaminobenzidine and 0.002% H_2O_2 in 0.05 M Tris-HCl for 10–20 min. Stained sections were dehydrated in alcohol and xylene, mounted in Permount and viewed under bright field and interference contrast optics. *a*, Immunoperoxidase staining of sensory neurone cell bodies in sections of cervical dorsal root ganglia. Immunoreaction product is present throughout the cytoplasm although membrane associated staining can also be observed. In many sections, large neuronal cell bodies appeared more densely stained than small neurones. Stained fibres can also be seen within the section although satellite and other non-neuronal cells appear unstained. Scale bar, 50 μ m. *b*, Immunofluorescent staining of rat dorsal root ganglion neuronal cell bodies, grown in dissociated cell culture for 10 days. Dorsal root ganglia were removed from rat embryos at 19 days of gestation, incubated with 0.05% trypsin and 0.01% collagenase (Worthington type IV) for 20 min at 37°C and dissociated by trituration. Cells were plated onto collagen and fibronectin-coated 35-mm tissue culture dishes in Ham's F12 medium containing 7.5% heat-inactivated rat serum, 4% 17-day rat embryo extract, 2 mM glutamine, essential vitamins, 5,000 U ml⁻¹ penicillin and streptomycin, 44 mM glucose and 1 μ g ml⁻¹ purified nerve growth factor. Cultures were fed every 2–3 days and treated with cytosine arabinoside (10^{-5} M) for 24–28 h soon after plating. Cultures were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, and processed for immunofluorescence as described above. Patchy staining of two sensory neurone cell bodies can be observed with staining apparent in both the cytoplasm and in association with the surface membrane. Scale bar, 20 μ m.

six rats were sectioned in a tissue chopper and homogenized in 0.05 M Tris-HCl (pH 7.6) with a Dounce pestle, followed by centrifugation at 2,000g for 10 min. The resulting supernatant was then centrifuged at 50,000g for 30 min and the crude membrane pellet used for immunization.

Six-week-old female BALB/c mice were injected intraperitoneally with 0.2 ml of the membrane suspension (10 mg ml^{-1}) in phosphate-buffered saline (PBS pH 7.4) emulsified 1:1 with Freund's complete adjuvant. Injections were repeated twice at 14-day intervals. Three weeks later a final injection was performed, without adjuvant, and the spleens from immunized mice were removed after a further 3 days. Lymphocytes were fused¹⁴ with X63.Ag 8.653 myeloma cells¹⁵. Hybrid clones were screened after 10 days and positive wells were cloned three times by limiting dilution.

Hybridoma supernatants were screened by indirect immunofluorescence using fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse IgG on 30- μ m transverse sections of the dorsal root ganglia and spinal cord of rats fixed by perfusion with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Of 288 hybridoma supernatants examined, 24 exhibited positive staining of neuronal cell bodies and an additional 4 stained radial glial fibres. One supernatant, designated CE5, labelled dorsal root ganglion neurones (Fig. 1a), motoneurone cell bodies (Fig. 2c) and large-diameter neurones within laminae IV and V of the dorsal horn with little or no staining of neuronal cell bodies in laminae I–III. Numerous neural processes were also observed in the spinal grey matter although there was little or no labelling of the white matter. Hybrid cells secreting antibody CE5 were recloned three times and all subsequent studies were performed on tissue culture supernatant. Antibody CE5 was found to be of the 1gM λ subclass when tested by indirect immunofluorescence using subclass-specific antisera or after SDS-polyacrylamide gel electrophoresis of ³⁵S-methionine-labelled immunoglobulin (gels run in reducing conditions with known 1gG and 1gM markers¹⁶).

Subpopulations of mammalian neurones stained by CE5

The distribution of cells labelled by CE5 was determined by visualizing antibody-binding sites with FITC- or peroxidase-conjugated rabbit anti-mouse IgG (as detailed in Fig. 1 legend).

In sections of adult rat dorsal root ganglion, the cytoplasm and external membrane of sensory neurones was labelled, whereas non-neuronal cells were not (Fig. 1a). A similar distribution and selectivity of labelling were seen in dorsal root ganglion cells grown in dissociated culture (Fig. 1b). Although insufficient CE5 was bound to the surface of viable neurones to enable detection by indirect immunofluorescence¹⁷, sufficient antibody was bound to activate complement and mediate lysis of >90% of sensory neurones. Significantly, non-neuronal cells in these cultures were unaffected by exposure to CE5 plus complement, consistent with the failure of CE5 to label non-neuronal cells by indirect immunofluorescence.

In 30- μ m transverse sections of adult rat cerebellar cortex, Purkinje neurones were the only cells labelled (Fig. 2a) and showed intense staining of both the soma and dendritic tree, with occasional axonal labelling. Throughout the cerebellar cortex, staining was observed in the cell bodies of pyramidal-shaped neurones within layer V (Fig. 2d) and their basal and apical dendrites (Fig. 2e). The cell bodies of pyramidal-shaped neurones within layers II and III were faintly stained in some but not all cortical areas. Within the hippocampus, occasional pyramidal cell bodies and their dendrites were stained, as were the majority of granule cell bodies and mossy fibres in the dentate fascia. In the olfactory bulb, labelling was observed in mitral cell bodies and their dendrites projecting through the external plexiform layer and terminating in superficial glomeruli (Fig. 2f), while the cell bodies of intrinsic olfactory neurones were unstained. A complex pattern of neuronal labelling with CE5 was observed in other regions of the central nervous system (to be described in detail elsewhere), although staining in all

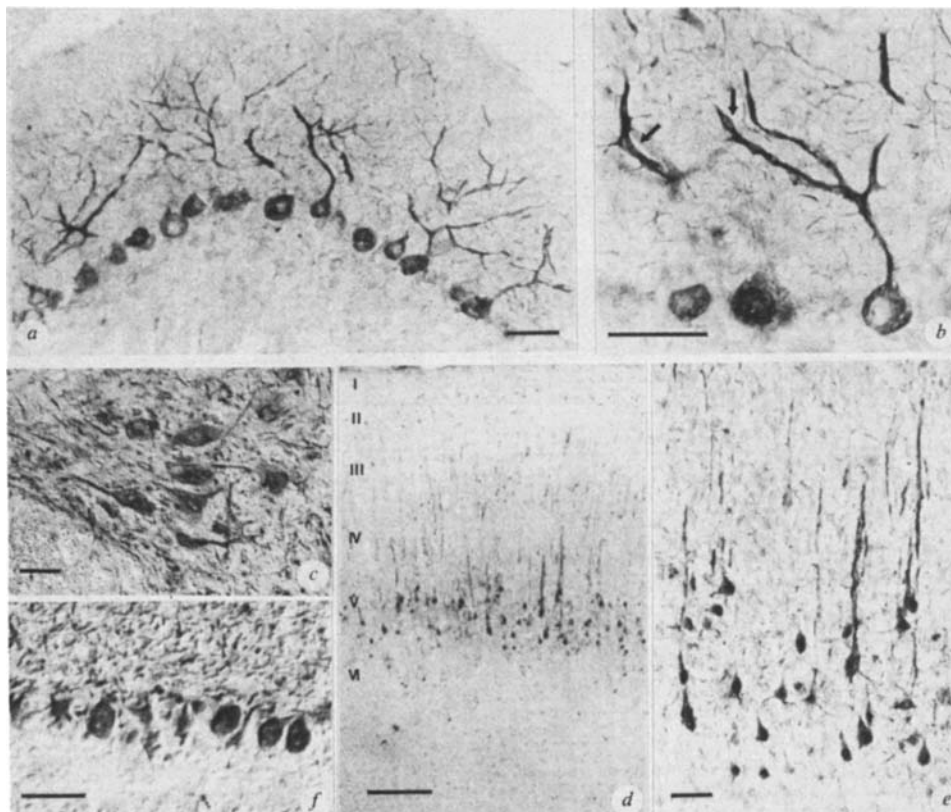


Fig. 2 Immunocytochemical localization of neurons in the rat central nervous system labelled with CE5. Immunoperoxidase staining procedures were carried out as described in Fig. 1 legend. *a*, Selective labelling of Purkinje neurone cell bodies and dendrites in rat cerebellar cortex. Scale bar, 100 μ m. *b*, High power micrograph showing the cylindrical labelling pattern (arrows) in oblique sections through Purkinje neurone dendrites, suggesting an association of the antigen with the surface membrane of the dendrite. The Purkinje neurone cell body, however, is labelled throughout the cytoplasm. Labelled axons can occasionally be seen projecting through the granular layer. Scale bar, 100 μ m. *c*, Staining of the cell bodies and initial processes of motoneurons, and other neuronal elements within the ventral horn of the rat cervical spinal cord. Scale bar, 50 μ m. *d*, Selective labelling of neurons in or near layer V of the rat occipital cortex. Dendrites can be seen projecting to more superficial layers. Scale bar, 200 μ m. *e*, High power micrograph showing the pyramidal appearance of labelled cell bodies within rat occipital cortex. Numerous superficial dendrites are stained and also occasional radial dendrites projecting within layer V. Scale bar, 50 μ m. *f*, Immunoperoxidase staining of mitral cell bodies and dendritic profiles in the olfactory bulb. Scale bar, 50 μ m.

regions was confined mainly to neurones with axons projecting beyond their nucleus of origin.

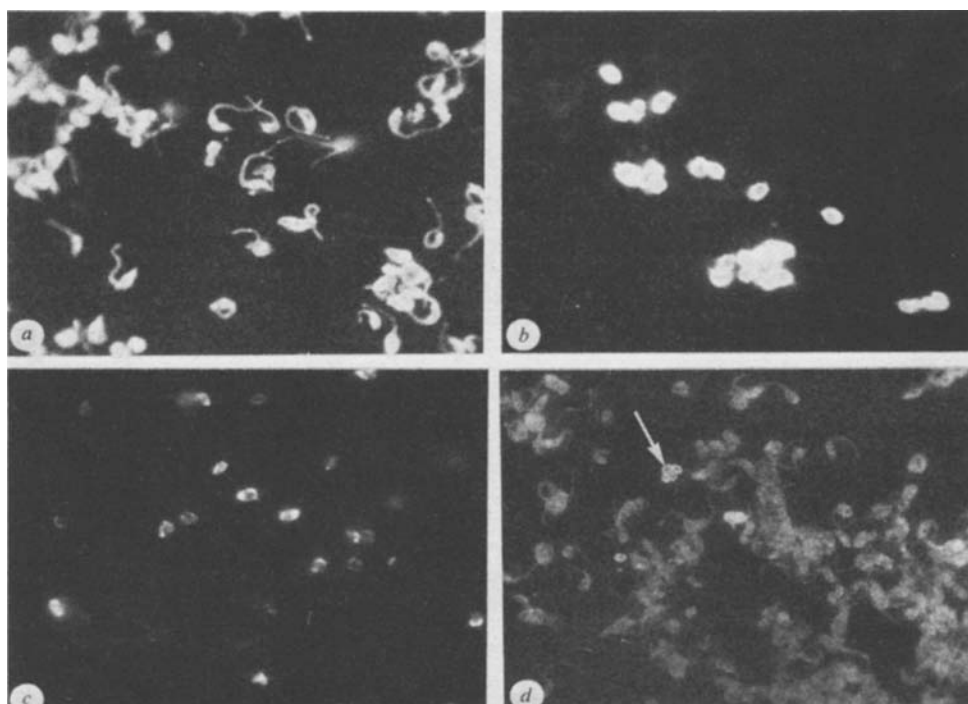
The specificity of neuronal labelling provides few clues, in itself, to the functional significance of CE5-defined antigens. Antigenic expression is clearly unrelated to the transmitter specificity of individual neurones and occurs in neurones derived from both the neural tube and neural crest. Other monoclonal antibodies recognizing subpopulations of vertebrate and invertebrate neurones reveal a completely different distribution of labelled cells¹⁷⁻²¹. In addition, although cerebellar Purkinje neurones and some but not all of the central

neurones stained by CE5 are also stained by polyclonal antisera directed against the calcium-binding proteins calmodulin²², parvalbumin²³ and vitamin D-dependent calcium-binding protein²⁴, CE5 did not react with any of these proteins. Antibody CE5 therefore seems to define a novel antigenic determinant in the mammalian nervous system.

Staining of trypanosomes by CE5

Of 11 monoclonal antibodies raised against rat neurofilaments, neuroglial cells or other neural antigens, only CE5 and its subclones stained formaldehyde-fixed *T. cruzi* when examined

Fig. 3 Staining of *T. cruzi* (Y Strain) organisms with CE5 monoclonal antibody. *a*, Formaldehyde-fixed epimastigotes showing homogeneous cytoplasmic fluorescence. *b*, Formaldehyde-fixed amastigotes showing bright fluorescence associated with the surface membrane. *c*, Viable amastigotes stained in suspension; living epimastigotes gave no reaction with CE5. *d*, Batch culture of epimastigotes containing a low percentage of amastigotes, after formaldehyde fixation. Epimastigotes gave typical cytoplasmic fluorescence whereas in amastigotes (arrowed) the antigen detected by CE5 is limited to the cell-surface membrane. As a specificity control, parasites were also treated with an IgM monoclonal antibody against dinitrophenol or with ascitic fluid containing MOPC 104E myeloma IgM. Positive staining was only observed with CE5.



by indirect immunofluorescence using FITC-conjugated anti-immunoglobulin serum. Epimastigotes of *T. cruzi* (Y strain) and *Herpetomonas samuelpessoai* were obtained from culture in Warren's medium²⁵. Non-motile amastigotes of *T. cruzi* were obtained from cultures of a murine thigh muscle tumour (S2) infected with trypomastigotes derived from the plasma of acutely infected mice. *T. brucei* trypomastigotes were isolated from the plasma of acutely infected rats. Whereas both epimastigotes and amastigotes of *T. cruzi* exhibited bright immunofluorescent staining (Fig. 3a, b), *T. brucei* and *H. samuelpessoai* showed no staining with CE5.

In addition, the various developmental stages of *T. cruzi* exhibit differential staining patterns with CE5. Paraformaldehyde-fixed epimastigotes reacted with CE5 to produce a homogeneous cytoplasmic staining (Fig. 3a) whereas staining of fixed amastigotes was only associated with the cell-surface membrane (Fig. 3b). The significance of this observation in relation to the manifestation of Chagasic symptoms would be greatly enhanced if this antigen was present on the surface of the parasite and thus directly available to the immune response. To examine this possibility, viable amastigotes and epimastigotes were incubated sequentially with CE5 and FITC-conjugated rabbit anti-mouse IgM. The surface membrane of viable amastigotes exhibited a bright immunofluorescence (Fig. 3c) whereas no staining was observed on the surface of viable epimastigotes. In the conditions of batch cultures used in these experiments, some epimastigotes showed spontaneous transformation into amastigotes. These viable amastigotes also exhibited intense membrane staining (Fig. 3d).

CE5-defined antigens and Chagas' disease

In the peripheral nervous system, one of the main histological features associated with *T. cruzi* infection is periganglionitis and degeneration of intrinsic myenteric neurones in the gastrointestinal tract⁵. In fixed whole-mount preparations of guinea-pig submucosal plexus²⁶ treated with CE5, clusters of neuronal cell bodies exhibited bright immunofluorescence within the cytoplasm and external membrane (Fig. 4a). Staining of neuronal processes within the submucosal plexus was also observed. However, without prior removal of the extrinsic sensory nerves projecting to the gut, it is not possible to determine whether these fibres originate from enteric neurones or from vagal and spinal sensory neurones.

The acute and chronic phases of Chagas' disease are characterized by the degeneration of striated muscle fibres in the heart, whereas skeletal muscle does not appear to be affected²⁷. Staining of cardiac muscle fibres appeared as intense cross-striations (Fig. 4c). The spacing between stained bands was 2.2–2.4 μm and sequential examination under bright field and phase contrast optics revealed staining located at the junction of A and I bands. The same pattern of staining was observed in glycerinated muscle fibres, suggesting that the antigen is closely linked to structural proteins in the muscle fibre. In contrast, CE5 did not stain skeletal muscle fibres removed from the rat tibialis anterior muscle.

The antigenic determinants recognized by CE5 are also present in the human nervous system; Purkinje neurones were stained selectively in postmortem human cerebellum (Fig. 4c). Although little is known about the central neuropathology in patients suffering from Chagas' disease, the only central neurones so far reported to undergo degeneration are cerebellar Purkinje neurones⁵.

Antigens recognized by CE5

The staining of Purkinje neurones in rat cerebellum could be abolished by preabsorbing CE5 hybridoma supernatant with an equal volume of formaldehyde-fixed *T. cruzi* epimastigotes, confirming that a common antigenic determinant is therefore expressed by mammalian neurones and *T. cruzi* parasites. If this determinant is relevant to the neuropathology associated with Chagas' disease, the serum of mammalian hosts infected

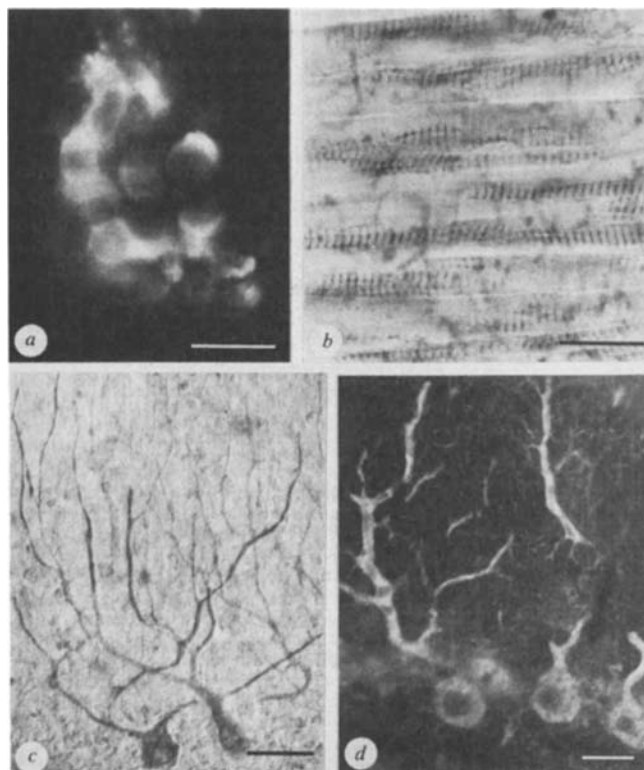


Fig. 4 CE5 labelling of mammalian cells known to degenerate in Chagas' disease. *a*, Immunofluorescent labelling of neuronal cell bodies in ganglia located in the submucosal plexus of the guinea pig. Whole-mount preparations of the guinea pig submucosal nerve plexus²⁶ were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer for 30 min, washed and processed for indirect immunofluorescence as described in Fig. 1 legend. Scale bar, 50 μm . *b*, Immunoperoxidase labelling of cross-striations in cardiac muscle fibres in 30- μm sections of formaldehyde-fixed rat heart exposed to CE5. Consistent staining of cross-striations can be observed. Apparent staining in the longitudinal axis reflects folds in the muscle fibre. Scale bar, 50 μm . *c*, Interference contrast micrograph showing immunoperoxidase staining of two Purkinje neurones in human cerebellar cortex. Human postmortem cerebellar cortex was obtained within 12 h of death and fixed by immersion for 24–48 h in 4% paraformaldehyde, washed for 24 h and processed for immunocytochemistry as described in Fig. 1 legend. Note the absence of staining of other neurones in the molecular layer. Scale bar, 100 μm . *d*, Immunofluorescent staining of rat cerebellar Purkinje neurones after exposure of cerebellar sections to sera obtained from mice infected 90 days previously with *T. cruzi* parasites. Sera from infected mice were diluted 1:10 with PBS and applied to cerebellar sections for 18–24 h at 4°C. After extensive washing, sections were incubated with FITC-conjugated anti-mouse immunoglobulin (Miles) diluted 1:50 for 30 min at room temperature, washed and mounted on glass slides. Intense staining of the cell bodies and dendrites of the Purkinje neurones can be seen, with less intense staining of other cellular elements in the molecular layer. Scale bar, 50 μm .

with *T. cruzi* might be expected to contain circulating antibodies directed against the same determinant. Sera from mice infected with *T. cruzi* for 14, 90 or 210 days were therefore used to stain sections of rat cerebellum. An intense labelling of the cell bodies and dendrites of Purkinje cells was seen, with additional, weaker staining of other cells in the molecular and granular layers (Fig. 4d). Sera from uninfected mice gave no specific staining.

The determinants in mammalian cells and *T. cruzi* that are recognized by CE5 were analysed using the immune blotting technique of Towbin *et al.*²⁸. All rat neuronal tissue, of central or peripheral origin, produced a similar pattern (Fig. 5, lanes *c*, *d*). A predominant immunoreactive band with an apparent molecular weight (M_r) of ~60,000 was observed together with a minor band of M_r ~32,000 and very faint bands M_r > 70,000.

Extracts of cardiac muscle revealed an intense band of M_r ~20,000 (Fig. 5, lane *e*) whereas skeletal muscle showed no stained bands, in accord with the results found by immunocytochemistry. Several very faint bands were also

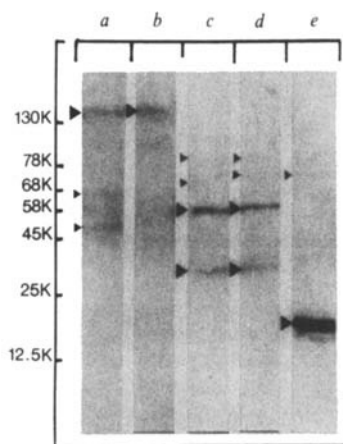


Fig. 5 Autoradiographic localization of proteins stained by CE5 after polyacrylamide gel electrophoresis and electrophoretic transfer of proteins onto nitrocellulose. Samples (50–100 µg) were boiled for 2 min in 10% SDS containing 2% β-mercaptoethanol and electrophoresed in 10% polyacrylamide gels in the presence of SDS. After electrophoresis, the gel was blotted onto a nitrocellulose sheet for 2 h at 12 V. The sheet was then incubated overnight in PBS containing 3% haemoglobin. CE5 tissue culture supernatant diluted 1:10 in PBS–haemoglobin was then applied to the sheet for 60 min. After six washes in PBS, ¹²⁵I-labelled rabbit anti-mouse globulins (5×10^6 c.p.m.) in PBS–haemoglobin was applied to the sheet for 60 min, and the sheet then washed a further six times in PBS. The dried sheet was exposed for 5–15 days at -70°C using Kodak X-omat H film. Lane a, epimastigotes; lane b, amastigotes; lane c, 7-day rat dorsal root ganglia; lane d, rat cerebellum; lane e, rat heart. The positions of molecular weight standards are indicated.

observed in extracts of liver, kidney and testis and a very faint staining reaction was found in these tissues by immunocytochemistry. The staining patterns produced by extracts of *T. cruzi* epimastigotes and amastigotes were distinct, with bands in a molecular weight range of 50,000–100,000 (Fig. 5, lanes a, b). Extracts of *T. brucei*, however, exhibited no immune staining, again in agreement with immunofluorescence studies.

The complex banding pattern shown by CE5 may reflect a common amino acid sequence in distinct proteins, breakdown products of a single large protein or a carbohydrate moiety common to several glycoproteins. Exposure of rat cerebellar sections to neuraminidase, mannosidase or periodate oxidation at pH 7.4 (ref. 29) did not affect the staining of Purkinje neurones by CE5. Similarly, incubation in the presence of 100 mM α-methyl mannoside, sucrose, galactose or mannose did not reduce the binding of CE5 to Purkinje neurones. It is therefore probable that the CE5-defined antigen is a polypeptide structure present in several different proteins which share a common epitope. Such complexity in staining patterns obtained with monospecific antibodies is not unique. Monoclonal antibodies to glial fibrillary acidic protein³⁰, and to Thy 1.1 (ref. 31) have recently revealed unsuspected antigenic cross-reactivity between distinct proteins.

Conclusions

Although previous serological studies^{10–13} have suggested that mammalian neurones, cardiac muscle and *T. cruzi* organisms might share common antigenic determinants, the identification of these antigens using monoclonal antibodies provides a more reproducible basis for future studies. At present, we have no direct evidence that the cross-reactive antigenic determinant recognized by CE5 is involved in the generation of Chagasic lesions. Previous experimental measurements of cell-mediated immunity against cardiac and neuronal antigens showed that a significant degree of autoimmunity was attained only after acute phase degeneration of cardiac neurones had occurred⁷. The late appearance of anti-self-reactivity was taken as evidence that autoimmunity was the result of the pathogenic process and so could not be involved in its initiation. Although autoimmunity provoked by cross-reactive structures might still have a secondary role in the development of Chagasic lesions, the description of anti-neuronal autoantibodies soon after infection and coincident with neuronal destruction³² suggests that an antibody-mediated mechanism might initiate or amplify acute- and chronic-phase lesions in cardiac or neuronal cells.

The availability of CE5 antibody should allow a direct assessment of the role of cross-reactive antigens in the development of Chagasic lesions. For example, proteins expressing the antigen could be purified from mammalian cells or *T. cruzi* organisms using affinity columns and injected in mice. The appearance of Chagasic lesions would provide strong evidence for their involvement in the disease. It will also be important to determine the effect of central or peripheral administration of CE5 antibody to normal or *T. cruzi*-infected mice. In infected mice, it is difficult to predict the outcome of CE5 treatment: the result might be parasite and/or antigen clearance with consequent disease amelioration, or alternatively increased neuronal and cardiac damage with disease exacerbation.

The present report may have important implications for the clinical study of Chagas' disease. If the similarity in neuronal staining in rat and human brain extends beyond the cerebellum, it may be appropriate to examine the central neuropathy associated with Chagas' disease in more detail.

Only 1 of 11 antibodies raised against mammalian neural antigens has revealed a cross-reactivity with *T. cruzi*. Further studies using monoclonal antibodies directed against peripheral and central neurones will reveal whether other shared determinants exist and may also provide a greater variety of immunological probes with which to study Chagas' disease.

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Regulation of metallothionein–thymidine kinase fusion plasmids injected into mouse eggs

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A plasmid was constructed with the promoter/regulatory region of the mouse metallothionein-I gene fused to the structural gene of herpesvirus thymidine kinase. When mouse eggs were microinjected with this plasmid and incubated with cadmium (a natural inducer of metallothionein gene transcription) thymidine kinase activity increased ~10-fold compared with control eggs not exposed to cadmium. Analysis of a set of deletion mutants revealed that the minimum sequence required for cadmium regulation lies within 90 nucleotides of the transcription start site.

METALLOTHIONEINS (MTs) are ubiquitous proteins of vertebrates and invertebrates that bind heavy metals and are thought to be involved in zinc homeostasis and resistance to heavy metal toxicity¹. The metallothionein-I (MT-I) gene is controlled at the transcriptional level by heavy metals and by glucocorticoid hormones^{2–4}. To ascertain which sequences are involved in transcriptional regulation, an assay system is required which allows faithful transcription and responds to regulatory molecules. There are currently three basic approaches: cell transformation^{5–9}, cell-free transcription^{10,11} and microinjection of DNA into cells. Plasmids containing intact eukaryotic genes or various mutations created from them *in vitro* have been injected into the germinal vesicle of frog oocytes to assess the nucleotide sequences required for synthesis of functional mRNAs encoding histones¹², globin¹³, ovalbumin¹⁴ and thymidine kinase¹⁵. Mouse oocytes have been used to study the expression of injected 5S ribosomal genes¹⁶ and mammalian tissue culture cells have been transformed by microinjection of DNA^{17,18}. DNA injected into fertilized mouse eggs may be retained in the adults that develop after reimplanting the eggs into foster mice and in some cases the foreign DNA is expressed^{19–23}. However, use of mammalian cells for quantitative analysis of transient expression of microinjected plasmids has not previously been reported.

Here we establish the usefulness of microinjecting plasmids into mouse ova as a convenient system for studying transcriptional regulation. The major advantages of this system are that it is homologous to the promoter under study, which should optimize the chances for faithful transcription and regulation, and it is rapid compared with cell transformation. To distinguish transcription originating from the injected genes from that of the endogenous genes, we have fused the mouse MT-I gene promoter and 5'-flanking region to the thymidine kinase (TK) gene of herpes simplex virus (HSV). Viral TK is readily assayed and can be distinguished from endogenous TK^{24,25}. Our results indicate that the MT-I promoter in the fusion plasmid is as effective as the normal HSV TK promoter in allowing synthesis of functional TK when injected into fertilized mouse ova. Furthermore, the MT-I promoter and adjacent DNA sequences respond to Cd and regulate the production of viral TK.

Expression of viral thymidine kinase in mouse ova

In the first experiments, TK activity was monitored by measuring the incorporation of ¹²⁵I-deoxycytidine (¹²⁵I-dC) into DNA. This assay exploits the fact that the HSV TK can phosphorylate ¹²⁵I-dC whereas the mammalian enzyme cannot²⁴; hence, the

Table 1 HSV TK activity in mouse fertilized one-cell eggs injected with various plasmids

Plasmids injected	Molecules of plasmid injected per egg	Incorporation of ¹²⁵ I-dC into DNA* (c.p.m.)	Formation of ³ H-TMP† (c.p.m. × 10 ⁻³)
a			
None	—	99 ± 18	
pBR322	4,400	101 ± 13	
pMT	2,300	80 ± 10	
pTK	2,300	1,230 ± 310	
pMT-TK	1,600	2,860 ± 940	
pMK	2,000	2,900 ± 480	
pMK	200	470 ± 30	
pMK	20	180 ± 50	
b			
None	—		9.06 ± 0.64
pBR322	2,000		7.35 ± 0.17
	20,000		9.17 ± 1.1
pTK	2,000		94 ± 22
	20,000		870 ± 205
pMK	2,000		170 ± 21
	20,000		970 ± 155

Fertilized one-cell ova were flushed from the oviduct using Brinster's medium²⁸ on the morning of day 1 of pregnancy. Cumulus cells were removed from ova with hyaluronidase (300 U ml⁻¹), and the ova were washed free of debris and enzyme before manipulation. For injection, the ova were transferred to a depression slide in Brinster's medium containing 5 µg ml⁻¹ cytochalasin B and held in place using a blunt pipette while the tip of the injector pipette was inserted through the zona pellucida and vitellus and into the cell nucleus²³. The DNA solution in the injector pipette was slowly discharged into the nucleus using a syringe connected to a micrometer. The larger pronucleus (male) of the fertilized ovum was injected with ~2 µl of plasmid solution. The injection volume was estimated from the increase in diameter of the nucleus. After injection the ova were washed free of cytochalasin and returned to the same medium used for collection. When the injections were completed the ova were incubated for 22 h in 25 µl of medium²⁸. The plasmids injected were: pBR322, the vector plasmid into which the genes were cloned; pMT, which contains the mouse MT-I²⁶ gene; pTK, which contains HSV TK²⁷; pMT-TK, which contains both the MT-I and HSV TK genes²³; and pMK, which contains the promoter of MT-I fused to the HSV TK structural gene (Fig. 1).

* Values are c.p.m. ± s.e.m. of ¹²⁵I-dC (0.5 µCi; >700 Ci mmol⁻¹; NEN) incorporated into DNA by 25 ova during a 22 h incubation period.

† TK assays were performed in the following manner. After injection of the plasmid, the ova were incubated for 22 h in Brinster's medium then transferred to 20 µl of hypotonic buffer (10 mM KCl, 2 mM MgCl₂, 10 mM Tris-HCl, pH 7.4, 1 mM ATP, 10 mM β-mercaptoethanol, 50 mM ε-aminocaproic acid and 1 mg ml⁻¹ bovine serum albumin)²⁹. The cells were frozen and thawed three times and 20 µl of reaction mixture (150 mM Tris-HCl, pH 7.5, 10 mM ATP, 10 mM MgCl₂, 25 mM NaF, 10 mM β-mercaptoethanol) were added followed by 5 µl of water containing 5 µCi ³H-thymidine (80 Ci mmol⁻¹; NEN). The mixture was incubated for 2 h at 37 °C, and the ³H-TMP produced was measured by adsorption on to DE-81 paper and subsequent scintillation counting^{30,31}. The values are c.p.m. ± s.e.m. of ³H-TMP formed by 25 ova injected with the indicated number of plasmids and incubated for 22 h before assay.

background is very low. Table 1 compares TK activity measured after injecting a few thousand copies of plasmids pBR322, pMT (which contains a 3.8 kilobase (kb) MT-I gene fragment²⁶ inserted into the *EcoRI* site of pBR322), pTK (which contains a 3.5-kb HSV TK fragment²⁷ inserted into the *Bam*HI site of pBR322), pMT-TK (which contains both MT-I and TK genes²³) and pMK (which has the MT-I gene promoter and 5'-flanking region fused to the TK gene²³; the predicted DNA sequence around the fusion site is shown in Fig. 1). Table 1a shows that incorporation of ¹²⁵I-dC into DNA during a 22 h incubation was the same as background when pBR322 or pMT were injected, but increased at least 10-fold when plasmids containing an intact TK gene were injected. A similar high level of ¹²⁵I-dC incorporation was observed when pMK was injected. Significant incorporation of ¹²⁵I-dC was detected with as few as 20 molecules of pMK injected per ovum. Table 1a indicates, however, that incorporation of ¹²⁵I-dC was not linearly related to plasmid copy number injected; in other experiments, injection of 20,000

or 200,000 copies of pMK gave no more incorporation than injection of 2,000 copies. We suspect that these results reflect the limited capacity of the fertilized ovum to synthesize DNA; hence, when large amounts of plasmids are injected, DNA synthesis becomes rate limiting rather than conversion of I-dC to I-dCMP by viral TK.

To obviate this problem, we determined TK activity directly by measuring the conversion of ³H-labelled thymidine to ³H-TMP by homogenates of ova prepared 22 h after plasmid injection. With this assay, enzyme activity was nearly linear when up to 20,000 copies of plasmid were injected per ovum (Table 1b). Thus, the indirect assay with ¹²⁵I-dC provides sensitivity to small numbers of injected genes while the direct assay with ³H-thymidine is more useful when large numbers of genes are injected.

The results with pMK suggest that the MT-I gene promoter is substituting for the TK gene promoter to allow transcription of a functional TK mRNA. To ascertain whether the 5'-flanking region of the MT-I gene also allows regulation of transcription by heavy metals or glucocorticoids, the injected ova were incubated for 22 h with or without 50 μ M Cd or 100 nM dexamethasone before TK assay. Table 2 shows that Cd treatment results in a dramatic increase in TK activity when 200 or 2,000 gene copies were injected per ovum. When 20 gene copies were injected the response to Cd was low; however, when the more sensitive ¹²⁵I-dC technique was used, a 2.5-fold increase in TK activity was detected after Cd treatment (data not shown). The amount of TK activity obtained when plasmid pTK was injected was unaffected by Cd treatment (data not shown). These results indicate that the molecules responsible for heavy metal regulation of MT-I gene transcription are present in mouse ova and that the regulatory sequences are contained within the 1.7 kb of mouse DNA 5' of the *Bgl*II site. We have not detected any increase in TK activity in response to dexamethasone treatment using either assay. This may be due to the absence of functional glucocorticoid receptors, lack of necessary DNA sequences, or inappropriate chromatin structure.

We attempted to eliminate gene activity by cutting the pMK plasmid between the MT-I promoter and the TK structural gene at the *Bgl*II site (see Fig. 1). Some of the linearized molecules were treated with DNA polymerase I (Klenow fragment) to fill in the 'sticky ends' generated by *Bgl*II. In both cases the linearized molecules were separated from any uncut molecules or nicked circles by agarose gel electrophoresis. Table 3 shows that despite the separation of MT-I promoter from the TK structural gene, the injected ova still produced a high level of TK activity, whether or not the DNA ends were blunted with DNA polymerase I. Furthermore, the TK activity was greatly enhanced by Cd treatment. A likely explanation for these results is that the mouse ovum has the requisite enzymes necessary to re-ligate the linearized pMK plasmid. Comparison of the TK activity generated with different dosages of plasmids indicates that the activity obtained with the blunt-ended fragment is essentially the same as with supercoiled plasmid (compare Tables 2 and 3), suggesting that re-ligation is very efficient. Analysis of DNA injected into eggs by agarose gel electrophoresis and Southern blotting confirms that linear DNA molecules become re-ligated and supercoiled (data not shown).

To examine this phenomenon further, the pMK plasmid was cut to yield individual 'regulatory region' (*Pst*I to *Bgl*II) and 'structural gene' (*Bgl*II to *Pvu*II) fragments (see Fig. 1, inner circle). Each fragment was injected separately and in combination, as shown in Table 3. When high levels (4,000 per nucleus) of the separate fragments were injected no significant increase in TK activity above control levels was observed. Furthermore, injection of 4,000 molecules of both fragments did not result in demonstrable TK activity. However, re-ligation of the fragments *in vitro* followed by injection resulted in high levels of TK activity which were further elevated by Cd treatment. Injection of higher levels (24,000 molecules) of the separate fragments was also effective. Our interpretation of these results is that the

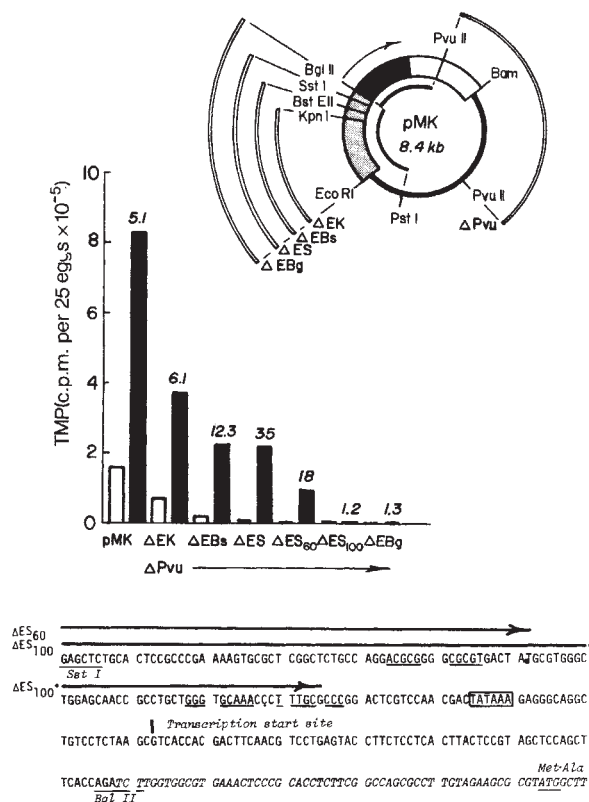


Fig. 1 TK activity generated from various 5'-deletion mutants of pMK. The plasmid is shown at the top. It was generated by restriction pMT-TK with *Bgl*II, which cuts just upstream of the initiation codon of both MT-I and TK genes, followed by ligation of the MT-I 5' sequences to the TK structural gene²³. The mouse DNA sequence of pMK is stippled, the HSV TK structural gene solid, the 3' HSV sequences are open, and the pBR322 sequences are shown as a solid line. The arrow indicates the direction of transcription. The *Pvu*II fragment of pMK was removed as shown by Δ Pvu; then a series of deletions were made that extend from *Eco*RI toward the 5' end of the TK gene. The *Sst*I, *Bst*EII and *Kpn*I sites lie ~150, 340 and 600 bp 5' of the transcription start site, respectively. *Bal*31 was used to extend deletions from *Sst*I site towards the TK gene. In each case an *Eco*RI linker was inserted and then ligated to the original *Eco*RI site to create deletions that extend from *Eco*RI to the positions indicated by the arrows ($\pm$ 5 bp) above the DNA sequence shown at the bottom; the end points were mapped by acrylamide gel electrophoresis of *Rsa*I/*Eco*RI digests. The MT-I sequence³³ between *Sst*I and *Bgl*II is shown fused to the HSV TK sequence³⁴ at the *Bgl*II site. Two palindromes, the TATAAA box, the transcription start site and the initiation codon for TK are indicated. Approximately 2,500 molecules of each plasmid were microinjected; TK activity was measured 22 h later as described in Table 1b legend. The results are the means of two experiments with each deletion mutant. Eggs were incubated with (solid histograms) or without Cd (open histograms) for 22 h before TK assay. The numbers over the histograms indicate the ratio of TK activity with and without Cd. The background TK activity of the eggs (2,000–5,000 c.p.m.) has not been subtracted. The activities of mutants Δ ES₁₀₀ and Δ EBg are not significantly different from background.

individual 'regulatory region' and 'structural gene' sequences do not have TK activity and that correct ligation of the separate fragments is inefficient compared with the circularization of the linearized plasmid.

Deletion mutants define promoter and regulatory sites

To define more precisely the mouse DNA sequences required for cadmium regulation we prepared a set of deletion mutants. The first step entailed deletion of a 3.0-kb *PvuII* fragment including the 3'-flanking region of the TK gene and non-essential pBR322 sequences; this fragment includes an *EcoRI* site that complicates construction of subsequent deletions. A set of deletions extending from the remaining *EcoRI* site towards the TK gene was then prepared by restriction with specific enzymes and re-ligation of blunted ends, as indicated in Fig. 1. To extend further towards the TK gene from the *SstI* site, the double-strand exonuclease *Bal31*, was used and an *EcoRI* linker inserted to facilitate mapping. The approximate (± 5 base pairs [bp]) end points of these deletions are indicated by arrows on the sequence at the bottom of Fig. 1. Figure 1 (histograms) shows that there was a progressive loss of constitutive as well as Cd-induced activity as the 5' sequences were deleted. TK activity measured in the absence of Cd was more severely decreased than in its presence; thus the ratio of activity with and without Cd increased (as indicated by the numbers over the histograms) until the minimal sequence required for Cd regulation was reached. The minimal sequence tested that preserves Cd regulation (ΔES_{60}) retains ~90 nucleotides of mouse DNA 5' of the transcription start site and includes a large palindrome centred at position -55. Deletions that extend into this palindrome (ΔES_{100}) or delete all the mouse sequences (ΔEBg) have no activity (<1.5 times background) and are not inducible by Cd. These data indicate that the 5' MT-I sequence carries both promoter and Cd regulatory sites. Although a minimal sequence can be described (exemplified by ΔES_{60}), additional sequences that potentiate both basal and induced activities clearly extend at least to the *KpnI* site, which is ~600 bp 5' of the transcription start site.

We conclude that fusion of the MT-I and HSV TK genes at their respective *BglII* sites results in a gene that is transcribed, presumably by RNA polymerase II⁴, and that the mRNA is translated into functional TK. Furthermore, the gene fusion is regulated by Cd in a manner similar to the endogenous MT-I gene. We have as yet been unable to dissociate a functional promoter from cadmium regulation. By 5'-deletion analysis we have identified a minimal control region that includes ~90 nucleotides 5' of the transcription start site. Plasmids that contain only 50 nucleotides 5' of the transcription start site are inactive plus or minus cadmium, despite the fact that they retain the TATAAA sequence that is generally required for transcription *in vitro*. These boundaries lie close to those defined by

Table 3 TK activity of restriction fragments derived from pMK

Plasmid treatment*	No. of DNA molecules injected	Formation of ³ H-TMP (c.p.m. $\times 10^{-3}$)†	
		-Cd	+50 μ M Cd
PMK restricted with <i>BglII</i>	2,000	92.6	1,000
PMK restricted with <i>BglII</i> and blunt-ended with DNA polymerase I	2,000	68.3	1,480
	400	50.8	514
	80	5.1	39.9
TK 'structural gene' fragment (<i>BglII</i> to <i>PvuII</i>)	4,000	6.8	6.5
MT-I 'regulatory region' fragment (<i>PstI</i> to <i>BglII</i>)	4,000	7.8	11.4
TK 'structural gene' and MT-I 'regulatory region' re-ligated <i>in vitro</i>	4,000 of each	105	407
TK 'structural gene' and MT-I 'regulatory region' injected as separate fragments	4,000 of each 24,000 of each	11.9 139	10.1 595
No plasmid injected	0	8.4	7.9

* Plasmid pMK was restricted with *BglII*; one-half of the sample was incubated with DNA polymerase I (Klenow fragment) in the presence of all four deoxyribonucleotide triphosphates. Then the DNA was electrophoresed on a 0.7% agarose gel; the linear band was localized by ethidium bromide staining (no supercoils or nicked circles were visible) and the DNA was eluted from the gel by the NaClO₄ method³². The TK 'structural gene' and MT-I 'regulatory region' fragments were isolated by cutting pMK with *PstI*, *BglII* and *PvuII* and separating the fragments on a 1% agarose gel and eluting them as described above. For re-ligation *in vitro*, 1.2 μ g of the *PstI*-*BglII* fragment (2,500 bp) was mixed with 0.9 μ g of the *BglII*-*PvuII* fragment (1,700 bp) in a 50 μ l reaction mixture with T4 ligase and incubated for 6 h at 15 °C. After ligation the mixture was extracted with SDS, phenol and chloroform, and the DNA was precipitated with ethanol.

† Ova were injected with the indicated number of DNA molecules and incubated for 22 h with (+) or without (-) 50 μ M Cd before TK assay as described in Table 1 legend.

McKnight *et al.*¹⁵ for the TK promoter. However, in contrast to their results, we find that sequences further upstream of -90 potentiate transcription. Some of these sequences allow transcription in the absence of Cd and may, therefore, represent additional promoters. Similar potentiating effects of upstream DNA sequences have been described for histone genes¹². The nature of the molecules involved in heavy metal regulation of MT-I genes is unknown, but they are apparently ubiquitous because we have demonstrated heavy metal regulation of MT-I genes in a wide variety of mouse organs², cell culture lines³ and now mouse oocytes and eggs. This feature may be useful for constructing expression vectors that can function in a wide variety of cell types. Another possibility we are exploring is that this regulatable gene can be introduced into mice by injecting plasmids into fertilized eggs and then implanting the eggs into pseudopregnant mice. The basic feasibility of this approach has been demonstrated²³ and preliminary results indicate that this fusion gene is indeed regulated in mouse liver.

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Table 2 Cd regulation of the amount of TK activity produced from pMK

No. of pMK plasmids injected	Formation of ³ H-TMP (c.p.m. $\times 10^{-3}$)*	
	Ova cultured without Cd	Ova cultured in 50 μ M Cd
2,000	190.0	2,400
200	25.3	270
20	8.3	12.2
0	8.5	8.8

Ova were injected with the number of pMK plasmids indicated as described in Table 1 legend. The ova were then randomly divided into two groups and incubated for 22 h with either 0 or 50 μ M Cd in the medium. TK activity was measured as described in Table 1 legend.

* Each value is the mean of ³H-TMP formed by 25 ova in two separate experiments.

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LETTERS TO NATURE

Association of PSR0740–28 with an H I shell in Puppis

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During a survey of the interstellar atomic hydrogen (H I) distribution in the Puppis region of the Milky Way, we detected numerous, sometimes expanding, H I shells and filaments, indicative of past energetic explosive events in this area of the Galaxy. One of these shells, GS241–01+15, appears to be physically associated with the young pulsar PSR0740–28. In particular, the pulsar is coincident in position with an H I hotspot on the rim of the shell, and there are two nearby non-thermal radio sources, one of which lies on the rim of the H I shell coincident with the position of the open cluster Hf11, the other lying near the centre of the shell. Both non-thermal sources have been previously identified as possible supernova remnants (SNRs)^{1,2}. Furthermore, velocity measurements of the peak emission from the hydrogen shell and of the 21-cm absorption spectrum of the pulsar offer strong confirmation that the pulsar and the H I shell lie at the same distance from the Sun. This result is of interest because very few pulsars have been linked to known supernova remnants, and only the Crab and Vela pulsars with any degree of certainty³. Whether the pulsar PSR0740–28 is the remnant of the supernova progenitor of the H I ring, or perhaps the remnant of an early supernova in a young stellar group which was formed by the expanding H I shell, is as yet unclear. Present data seem to support the latter interpretation, suggesting that PSR0740–28 may have resulted from a 'second-generation' supernova event.

The correlation between pulsar and shell positions was noted during an analysis of a 21-cm neutral hydrogen survey of a region of the galactic disk known as the Puppis window. This unique area of the Milky Way contains much less absorbing interstellar dust and associated gas than is found in more typical regions of the Galaxy. In fact, galaxies have been observed near the galactic equator^{4,5}. It is estimated that the total visual extinction out to the edge of the galactic disk in the direction of galactic longitude $l = 245^\circ$ is only four to five magnitudes⁴. This is a favourable longitude for study of large-scale galactic kinematics as it is near the middle of the third quadrant of galactic longitude where circular galactic rotation provides a near optimum ratio of radial velocity to distance. Hence, this region of the Galaxy is ideally suited for the comparison of optical and radio observations, extending out to much greater

distances than is normally feasible in other portions of the galactic disk. In addition, large holes and gaps in the H I gas distribution, in velocity space, make this region particularly suitable for the identification of shells and ring-like structures, as a lack of foreground and background gas along various lines-of-sight minimizes the confusion of sources which would otherwise result.

The radio observations were conducted on the 43-m National Radio Astronomy Observatory (NRAO) radio telescope at Green Bank, West Virginia during two observing sessions: 22–28 June 1979 and 12–19 May 1980. The area observed consists of a 61×61 grid of points in the sky, extending from galactic longitudes $l = 239^\circ$ to 251° and from galactic latitudes $b = -9^\circ$ to $+3^\circ$, every 0.2° in l and b . The half-power beamwidth of the telescope at the 21-cm wavelength corresponding to neutral hydrogen emission is 21 arc min. The main results of our survey, along with a detailed description of our data reduction procedure, will be presented elsewhere⁶.

The H I shell which we denote by GS241–01+15 (GS refers to galactic shell, followed by position and velocity information in the format $l + b + v_{\text{LSR}}$, where all velocities have been referred to the local standard of rest (LSR)) was previously noted by Heiles⁷ in his analysis of the Berkeley low-latitude survey of neutral hydrogen⁸, and labelled as GS242–01+11. The finer spatial and velocity resolution of the present survey accounts for the differences in position and velocity range reported for this object. The centre of this H I shell coincides with an extended ($1.2^\circ \times 1.2^\circ$) radio source G240.8–1.2 discovered in a 29.9-MHz continuum survey by Jones and Finlay, and tentatively identified as a SNR¹. The more recent 408 MHz maps of Haslam *et al.*⁹ contain a hint of this ring-like H I feature, though the correlation is far from exact.

The pulsar position ($l = 243.8^\circ$, $b = -2.4^\circ$) places it on the eastern edge of the H I shell, coincident with a fairly strong peak of 21-cm emission (H I cloud GC244–03+15) and just adjacent to another extended ($1^\circ \times 1^\circ$) non-thermal radio source G242.5–3.5 (see Figs 1 and 2)¹. Gomez-Gonzalez *et al.*¹⁰ detected strong absorption towards the pulsar in their observations with the Nançay radio telescope, having a velocity resolution of 12.7 km s^{-1} . Half of the pulsar signal is absorbed in their channel centred at $+19.5 \text{ km s}^{-1}$, indicating that the absorption extends to at least the centre velocity. No absorption at all is seen in the next channel, centred at $+32.2 \text{ km s}^{-1}$, indicating that the absorption has stopped before the lower velocity range of this channel, at $\sim +26 \text{ km s}^{-1}$. Thus absorption ceases between $+19.5$ and $+26 \text{ km s}^{-1}$. The highest velocity at which we can still recognize the shell GS241–01+15 in emission is at about $+20 \text{ km s}^{-1}$ where it blends in with strong generalized emission. This general emission peaks near $+20 \text{ km s}^{-1}$ but extends to about $+30 \text{ km s}^{-1}$ (see ref. 8). Since H I clouds which absorb the pulsar's signals must lie closer to us than the pulsar and H I clouds which do not appear in absorption presumably lie beyond the pulsar, the observed absorption indicates that the pulsar is at least as distant as the shell, and yet is not as distant as the numerous clouds causing the widespread H I emission at slightly higher velocities (and which are presumably lying just beyond the shell). As discussed

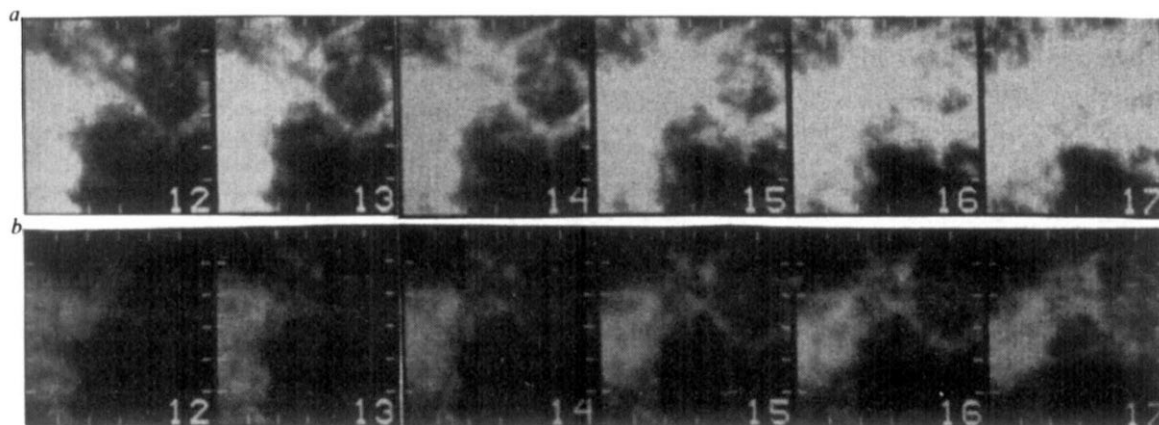


Fig. 1 Synthetic photographs of the constant-velocity neutral hydrogen (H I) distribution in the vicinity of the H I shell GS241–01+15 over the LSR velocity range +12 to +17 km s⁻¹. Tick marks are included in each map at 2° intervals at odd values of galactic longitude and latitude. Each map extends from $l=239^\circ$ to 251° (right-to-left) and from $b=+3^\circ$ to -9° (top-to-bottom). *a*, Highlights H I brightness temperatures over the range 5–50 K; *b*, over the range 25–105 K. The pulsar PSR0740–28 lies on the hotspot ($l=243.6^\circ$, $b=-2.6^\circ$) on the lower left rim of the shell.

by Weisberg *et al.*¹¹, bright H I emission clouds in the Milky Way rarely fail to provide significant absorption against extragalactic radio sources. Thus the pulsar PSR0740–28 lies not only on a hotspot of the H I shell in position, but also coincides with it in distance. The most recent distance estimate to this object, derived from a combination of dispersion measurements and H I absorption observations, is listed as 1.5 kpc (ref. 12), very nearly equivalent to our estimated kinematic distance of 1.4 kpc to the H I shell. We now consider possible physical mechanisms connecting the pulsar and the shell.

A summary of the observed and derived parameters describing the pulsar PSR0740–28, the H I shell GS241–01+15 and the H I cloud GC244–03+15 is given in Table 1.

PSR0740–28 is a very short-period pulsar (currently eighth-ranking of 330 pulsars by pulse period), whose measured period derivative implies a characteristic age of 1.58×10^5 yr (refs 12, 13). Striking similarities between PSR0740–28 and the Vela pulsar in terms of pulse shape and polarization properties have been noted¹⁴. At the time of its discovery¹⁵, it was noted that the pulsar PSR0740–28 was among the youngest detected without apparent association to a known SNR¹⁵.

To determine more precisely the relationship of the H I shell GS241–01+15 to the pulsar PSR0740–28, it is of interest to estimate the age of this presumed SNR for comparison. Unfortunately, most models^{16,17} relating the linear diameter of a SNR to its age are of questionable applicability in the present case as their results are appropriate for relatively young SNRs in the Sedov adiabatic expansion phase of their development. Our shell GS241–01+15, however, exhibits no appreciable variation in angular size with velocity, implying minimal expansion. This fact, coupled with the large linear size of the shell (~ 170 pc), leads us to believe that, if anything, GS241–01+15 is in transition from the isothermal expansion phase of its existence to the extinction phase, when the SNR begins to lose its identity as it merges with the interstellar medium.

Substituting typical values for initial supernova energy and ambient interstellar density into equation (1) of Herbst and Assousa¹⁸ yields an age in the range 10^6 – 10^7 yr for the shell GS241–01+15. Although the assumption of an unusually large supernova energy might bring the pulsar and shell ages into closer agreement, the indication is that such a large diameter H I shell has an age greater than that of PSR0740–28. Nonetheless, even disregarding the large uncertainties inherent in these estimates, such discrepancies between pulsar and SNR ages in reported associations (up to two orders of magnitude in some cases^{2,19}) have been said to have important implications in such fundamental areas as “supernova collapse theory, pulsar

acceleration mechanisms, and the birth rate and evolution of the galactic pulsar population”¹⁹.

Another factor to be considered is the displacement of the pulsar from the centre of the H I shell. Taking an angular separation between the pulsar and shell centre of $\sim 3.1^\circ$, and a distance of ~ 1.4 kpc, one finds that the pulsar is linearly displaced by about 75 pc from the centre of the H I ring. Assuming that the pulsar was originally at the centre of the H I shell, one may calculate its average annual proper motion, over a presumed lifetime of 1.58×10^5 yr. This value is found to be ~ 70 m arc s yr⁻¹, corresponding to a space velocity of ~ 500 km s⁻¹. Though large (even for such notorious high-velocity objects as pulsars), at least two pulsars have been

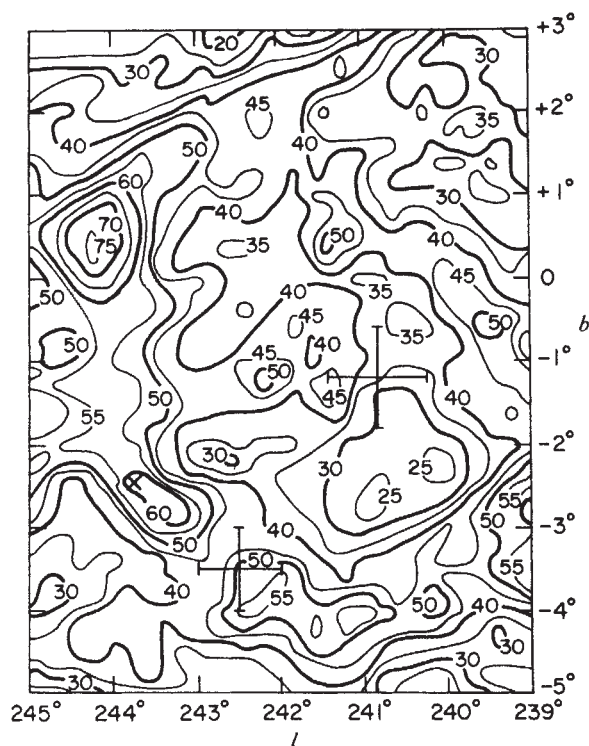


Fig. 2 Contours of 21-cm brightness temperature at an LSR velocity of +15 km s⁻¹ in the vicinity of the H I shell GS241–01+15. $\times$, The position of the pulsar PSR0740–28 ($l=243.8^\circ$, $b=-2.4^\circ$). $+$, The positions of the non-thermal radio sources G240.8–1.2 and G242.5–3.5; the length of the bars indicates the approximate angular extent of these features (from ref. 1).

Table 1 Observed and derived parameters describing GS241-01+15, GC244-03+15 and PSR0740-28

H I shell GS241-01+15 and H I cloud GC244-03+15											
	l_{ctr}	b_{ctr}	Δl	Δb	V_{min}	V_{max}	Δr	d	LD	$N_{\text{H I}}$	M
		(deg)			(km s ⁻¹)		(deg)	(kpc)	(pc)	(cm ⁻²)	(M _⊙)
Shell	241.	-1.	6.	8.	+7	+20	1.75	1.4	170	2.4×10^{20}	4.6×10^4
Cloud	243.6	-2.6	0.75	0.75	+7	+20	—	1.4	18	5.0×10^{20}	9.4×10^2
Pulsar PSR0740-28											
	l	b	P	$\dot{P}$	H I absorp.			d	Age		
	(deg)	(deg)	(s)	(10 ⁻¹⁵)	(km s ⁻¹)			(kpc)	(yr)		
	243.8	-2.4	0.1668	16.832	0 to +19.5			1.5	1.58×10^5		

Column headings for the H I shell and cloud are: l_{ctr} , b_{ctr} , galactic longitude and latitude of the centre of the radio features; Δl , Δb , approximate angular extent of the radio features in l and b (estimated to the nearest quarter degree); V_{min} , V_{max} , minimum and maximum LSR radial velocities between which the H I features are detected; Δr , the angular radial thickness of the H I shell (estimated to the nearest quarter degree); d , distance to the features from the Sun, calculated by applying a flat galactic rotation curve and using the LSR velocity of peak emission; LD , linear diameter of the H I features; $N_{\text{H I}}$, H I column density within the shell or cloud (cm⁻²); n , particle number density within the shell or cloud (cm⁻³), assuming 10% helium abundance by number; M , total mass contained within the shell or cloud expressed in solar masses, assuming 10% helium abundance by number. Pulsar column headings: l , b , galactic longitude and latitude; P , pulse period; $\dot{P}$, period derivative; H I absorp., LSR velocity of H I absorption; d , distance from the Sun; Age, characteristic age. All pulsar parameters are from ref. 12 except column 5 which is from ref. 10.

observed with transverse velocities in excess of 500 km s⁻¹ (refs 20, 21), and pulsar velocities up to several thousand km s⁻¹ have been repeatedly invoked to explain previously reported pulsar-SNR associations^{2,19}.

An alternate hypothesis regarding the origin of the pulsar is that the supernova which gave birth to PSR0740-28 was not the same one as that which gave rise to the H I shell. In other words, the pulsar has resulted from a relatively recent supernova event in a very young stellar group which was formed by the gradual expansion of the much older H I shell into an adjacent H I cloud, with subsequent shock-wave induced star formation. The presence of the pulsar in a hot spot on the H I shell, just adjacent to a non-thermal radio source, supports this interpretation. No obvious evidence of this second later supernova should be expected in our current H I observations, as the expected size of such a distant and young H I shell would be comparable to the beam size of the 43-m NRAO antenna. Such a second hypothesis supports the results of Morris *et al.*² in naming the non-thermal source G242.5-3.5 as the likely point-of-origin of PSR0740-28, while simultaneously explaining its proximity to the non-thermal source G240.8-1.2 and the extended H I shell GS241-01+15, remnants of a much earlier supernova.

The position angle of PSR0740-28 relative to G242.5-3.5 is 110°, and relative to G240.8-1.2 is 173°. According to Morris *et al.*², the intrinsic polarization vector for PSR0740-28 lies at 108° ± 4°, and the former source is the preferred point of origin for the pulsar, as it places the quantity (polarization angle minus position vector) close to the secondary peak (at 0°) in the probability distribution of this quantity as plotted for several pulsars. The angular separation between PSR0740-28 and G242.5-3.5 (~1.7°) also implies a more reasonable transverse velocity of ~250 km s⁻¹. A logical follow-up to this hypothesis would be proper motion studies of PSR0740-28. The proper motions mentioned above should be detectable, and offer the means of differentiating between the H I shell and G242.5-3.5 as the more likely origin of the pulsar. Note how cloud GC244-03+15 is apparently elongated along the line joining the pulsar to G242.5-3.5. The position of G242.5-3.5 also falls within the boundaries of the compact open cluster OC1-657 = Hf11 ($l = 242.41^\circ$, $b = -3.56^\circ$)²². Other open clusters which have been associated with SNRs are Tr18 (ref. 23) and NGC559 (ref. 24). Very little is currently known about Hf11, which is also listed as cluster number 3 in the compilation of van den Bergh and Hagen²⁵, and thus we cannot determine whether its age is consistent with the age of the H I shell. Clearly, a programme of photometry and spectroscopy on this cluster is necessary to determine its age.

Other clusters in the general vicinity which may be related to the H I shell GS241-01+15 are Tr9 ($l = 243.1^\circ$, $b = +1.2^\circ$) and NGC2453 ($l = 243.3^\circ$, $b = -0.9^\circ$). Fenkart and Binggeli²⁶ give distances and earliest spectral types of 2.23 kpc and b5 for

Tr9, and 1.49 kpc and b7 for NGC2453. A search for very young stars and molecular clouds in the vicinity of the pulsar and near other H I hotspots on the shell might provide further evidence for possible shock-wave induced star formation along the eastern rim of the H I shell (thus supporting the latter hypothesis of origin of the pulsar).

The coincidence in both coordinate position and distance of the pulsar PSR0740-28 and the neutral hydrogen shell GS241-01+15, in conjunction with the presence of two nearby non-thermal radio sources, G242.5-3.5 and G240.8-1.2, is strong support for the physical relationship of these two objects. It is not yet clear whether the pulsar and the H I shell originated in a single supernova event, or whether formation of the H I shell may have preceded the birth of PSR0740-28. The current data seem to indicate that PSR0740-28 may be the remnant of a second-generation supernova, in support of the conclusions of Morris *et al.*² regarding the relationship between the pulsar and the non-thermal source G242.5-3.5. Future observations, particularly proper motion studies, should clarify the situation.

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Overshoots in planetary bow shocks

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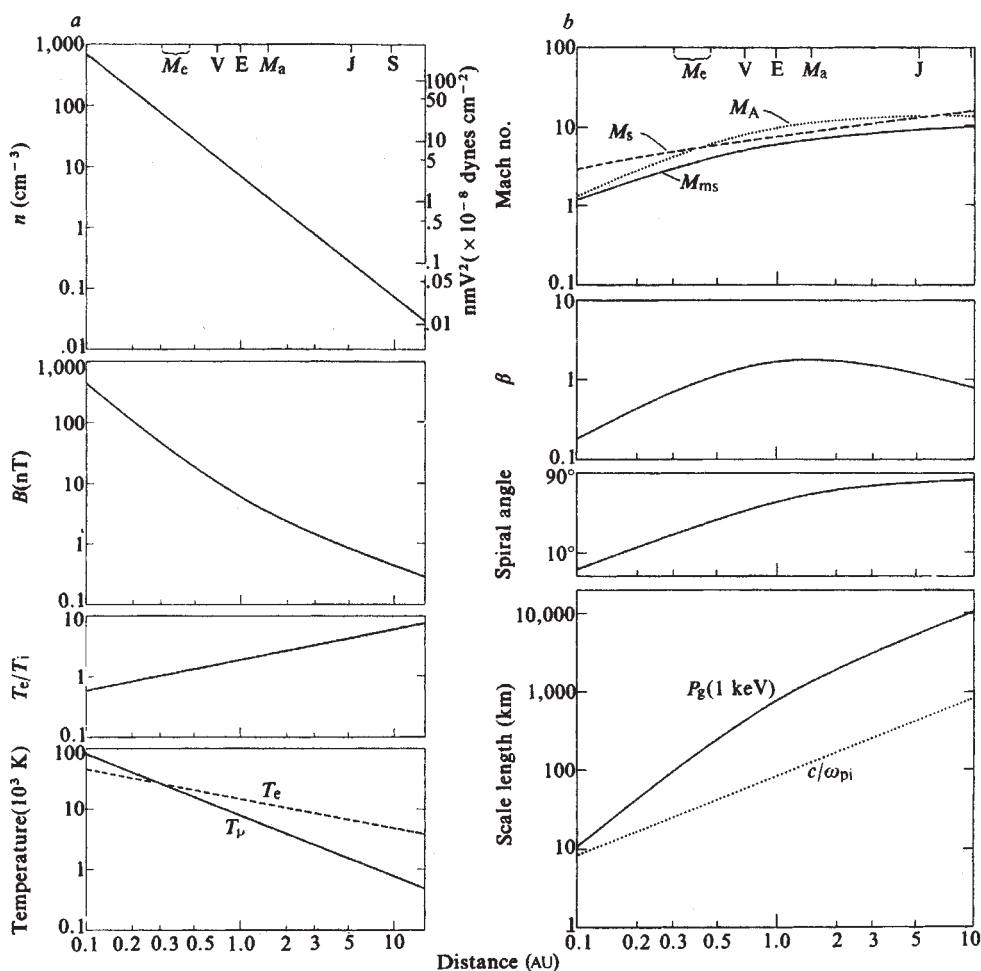
An overshoot in the magnitude of the magnetic field is shown here to be a consistent feature of supercritical collisionless shocks, Mach number ≥ 3 , throughout the Solar System. Although the variability of the solar wind at 1 AU allows us to study the Earth's bow shock over a wide range of β , the ratio of thermal pressure to magnetic pressure, the Mach number of the solar wind at 1 AU varies over a relatively narrow range. Data from Jupiter and Saturn allow us to study the bow shock at high Mach numbers rarely, if ever, observed at Earth. These combined planetary data show that the planetary bow shocks, at least as characterized by their overshoots, form part of a continuum, differences being dependent mainly on the varying solar wind conditions at each of the planets. The overshoot magnitude is found to increase both with β and magnetosonic Mach number. However, it is the radial variation of Mach number which causes the overshoot in the jovian and saturnian bow shocks to be much greater than in the terrestrial shock.

Collisionless bow shock waves exist in front of each planet probed by planetary spacecraft so far. These shocks both deflect the supersonic, or supermagnetosonic, solar wind around the planets and heat the solar wind. Part of the incoming solar wind is accelerated or 'reflected' by the shock back along magnetic field lines towards the Sun. This reflection process has been described geometrically by Sonnerup¹ but no detailed mechanism has yet been postulated to reflect the observed small fraction of the incoming ion beam. The change in the solar wind

plasma across the bow shock can be understood in magneto-hydrodynamic formalism according to the Rankine-Hugoniot equations². However, these equations do not describe the plasma physical processes by which the heating and deflection occur, the relative energization of ions and electrons, the heat flux represented by the 'reflected' ion beams, or the fine scale structure of the shock on scale lengths comparable to an ion gyro-radius or less. Investigation of the microphysics of collisionless shocks is necessary to understand these processes and to learn why in collisionless shocks a small fraction of the particles receives a large fraction of the energy. This behaviour is important, not just for understanding the creation of energetic particles in the Solar System, but also for understanding the generation cosmic rays outside the Solar System. It is quite likely that stellar, interstellar and even intergalactic shocks are producing cosmic ray beams analogous to the ion beams in planetary foreshocks.

Because of the dispersive nature of waves in a plasma one would expect damped wave trains either upstream or downstream from the shock front². What is generally observed in planetary shocks is a single approximately critically damped wave cycle or overshoot in the magnetic field strength. This overshoot was first noted by Heppner *et al.*³ in OGO 1 magnetic field records and later reported in ISEE 1 and 2 data^{4,5}, in which it was shown that while electron acceleration is coincident with the overshoot in field magnitude, ion thermalization is not complete until the overshoot has finished and the field returns approximately to its equilibrium or Rankine-Hugoniot value. There is reason to believe this overshoot is associated intimately with the ion 'reflection' process for unpublished ISEE magnetic field data indicate a scale length for the overshoot comparable to the ion gyro-radius, and the plasma data show ions reflected by the overshoot. These observations are supported by computer simulations which also demonstrate the ion gyration nature of the overshoot⁶.

Fig. 1 Typical solar wind parameters as a function of heliocentric distance. *a*, Number density and dynamic pressure (top panel), magnetic field strength, ratio of electron to proton temperature, and the electron and proton temperatures. *b*, Mach numbers, Alfvén, sonic and magnetosonic (top panel), β (ratio of thermal to magnetic pressure), the spiral angle (angle between interplanetary field and solar wind flow) and gyroradius of 1-keV proton and the ion inertial length. The labels at the top give the planetary positions.



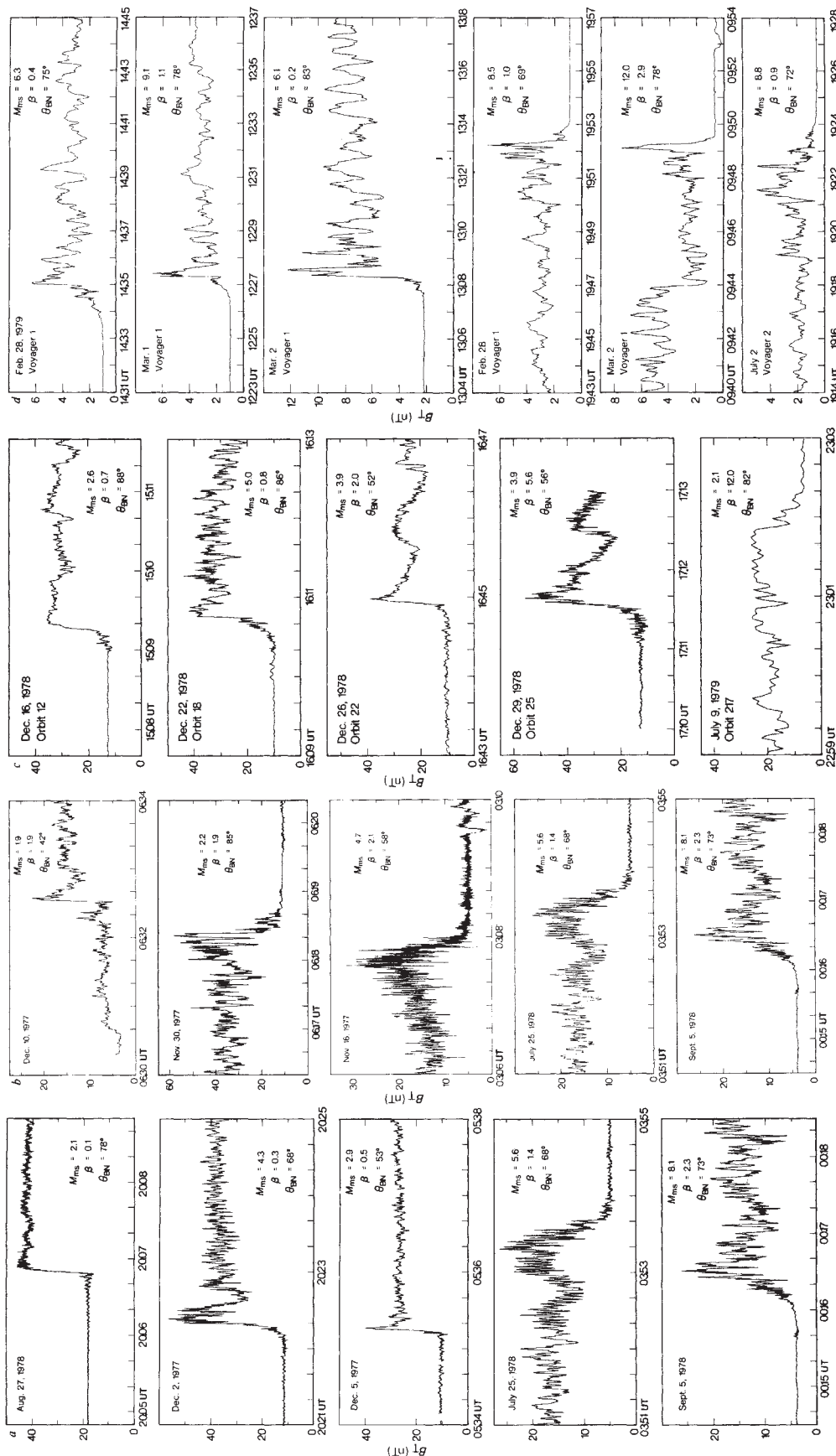


Fig. 2 Magnetic field profiles of the terrestrial bow shock as observed by the ISEE 1 magnetometer in a variety of solar wind conditions. *a, b* increases from top to bottom. *b*, Magnetosonic Mach number increases from top to bottom. *c*, Magnetic field profiles of the Venus bow shock in a variety of solar wind conditions. The top two panels contrast shocks for different Mach number. The middle two panels contrast shocks for differing β . *d*, Six crossings of the jovian bow shock as detected by Voyagers 1 and 2.

Although the existence of the overshoot in the terrestrial shock has long been known, its parametric variation with solar wind conditions has not been studied nor has its possible importance in the ion reflection mechanism been appreciated. We now consider the parametric variation of the overshoot with solar wind conditions by using both terrestrial and planetary

bow shock data. At the Earth the day-to-day variability of the solar wind and variation of the angle between the solar wind flow and magnetic field and the shock normal along the curved bow shock provide a wide range of plasma conditions. On the other hand, despite this variability the range of plasma conditions at 1 AU is limited and examples of shocks in some interesting

portions of parameter space, especially at high Mach numbers, are rare. This difficulty can be partially overcome through studying the bow shocks of the other planets as the variation of the solar wind properties with heliocentric distance places each of the planets in a different average environment.

This variation is illustrated in Fig. 1a, in which the dependence of various solar wind parameters is plotted as a function of heliocentric distance. The curves were constructed by taking estimates of typical values at the Earth (J. T. Gosling, personal communication), assuming a constant solar wind speed and scaling density as r^{-2} , magnetic field strength as r^{-1} ($r^{-2} + 1$)^{1/2}, proton temperature as r^{-1} and electron temperature as $r^{-1/2}$. In addition to these basic parameters we have studied the radial dependence of a set of physical quantities derived from them which have proved useful in classifying shock structure. These quantities are plotted against heliocentric distance in Fig. 1b. Included are plots of β , the spiral angle of the interplanetary field, the length scales of the ion gyroradius (ρ_s) and ion inertial length (c/ω_{pi}) and three Mach numbers. The Mach numbers are the Alfvén Mach number, the ratio of the solar wind velocity with respect to the Alfvén velocity, the sonic Mach number, the ratio of the solar wind velocity to the sound velocity and the magnetosonic Mach number, the ratio of the solar wind velocity to the magnetosonic velocity, or fast mode velocity. The magnetosonic velocity is calculated for propagation perpendicular to the magnetic field and is the square root of the sum of the squares of the Alfvén and sound velocities. All of the Mach numbers rise monotonically by factors ~ 3 from the orbit of Mercury to that of Saturn.

If the magnetosonic velocity were corrected for its dependence on angle of propagation to the magnetic field, the corrections would be small, especially at the larger heliocentric distances where the spiral angle of the interplanetary magnetic field becomes more perpendicular to the solar wind velocity. β first increases and then decreases with increasing distance, rising to its maximum just beyond the orbit of the Earth. The ion inertial length and gyroradius increase by one and two orders of magnitude respectively from Mercury to Saturn.

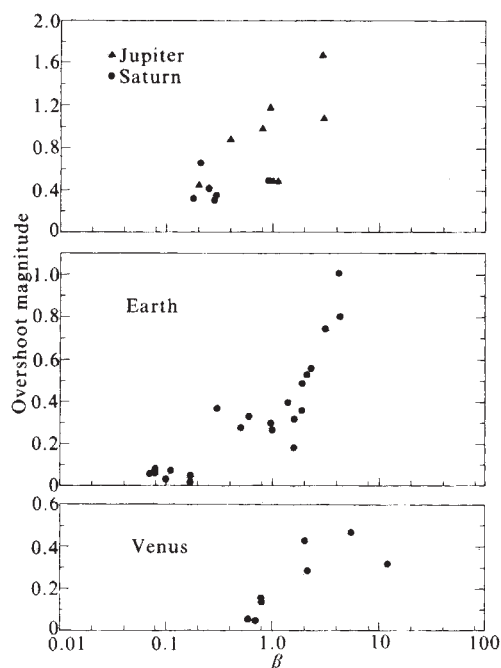


Fig. 3 Strength of the overshoot in the magnetic field immediately behind the shock as a function of β for Venus, Earth, Jupiter and Saturn. The overshoot magnitude is defined to be the maximum field strength minus the equilibrium average field strength some distance behind the shock normalized by this equilibrium average. The scale for the upper panel is double that of the bottom two panels.

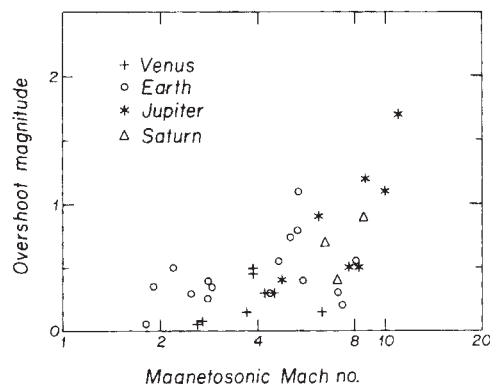


Fig. 4 The magnitude of the overshoot as a function of magnetosonic Mach number.

Experimental evidence that these predicted changes in plasma environment from planet to planet are reflected in the evolution of bow shock structure with heliocentric distance has been provided by Scarf *et al.*⁷ in their studies of the plasma wave spectra associated with various planetary bow shocks. The very low frequency spectrum across the bow shock of Saturn is distinctly peaked, whereas at Venus it decreases monotonically with increasing frequency. Data at the Earth and Jupiter show intermediate behaviour. Scarf *et al.*⁷ hypothesize that the nature of the bow shock undergoes a transition in the neighbourhood of a Mach number of 7–10.

We use magnetic field data obtained at Venus, Earth, Jupiter and Saturn to study the heliocentric evolution of bow shock overshoot and its dependence on solar wind parameters. We do not use data obtained at Mercury or Mars because of the paucity and/or unavailability of high resolution data. Figure 2a shows samples of ISEE 1 magnetic profiles⁸ across the terrestrial bow shock in a variety of solar wind conditions (Los Alamos Space Plasma Group, personal communication). On 27 August 1978 at low Mach number and β there is little if any post-shock overshoot in the magnetic field. However, at higher Mach number and β the overshoot is quite prominent. These shocks have been arranged in order of increasing β from top to bottom.

There is a variety of shock structure; the field can at times be quite irregular and the overshoot can be difficult to define unambiguously or to distinguish from 'normal' magnetosheath fluctuations. We have chosen a simple empirical parameterization of the overshoot and define the magnitude of the overshoot to be the difference between the equilibrium or average downstream field strength and the maximum field strength just behind the shock normalized by the equilibrium average field strength.

Figure 2b shows a set of shocks similar to those in Fig 2a arranged in order of increasing magnetosonic Mach number with β held fixed at ~ 2 . There seems to be an evolution in structure with changing Mach number but the evolution is partly masked by our inability to fix the angle between the upstream field and the shock normal. Furthermore, despite our examination of >1 yr of ISEE data we have been unable to separate clearly Mach number effects from β effects. Indeed the bottom two examples in Fig. 2a, b are the same cases.

Similar sets of shocks with contrasting solar wind parameters chosen from Pioneer Venus Orbiter data⁹ are shown in Fig. 2c. Solar wind conditions at Venus are similar to those seen at Earth, and the bow shock structure is similar although weaker than the terrestrial shock¹⁰. The top two panels of Fig. 2c contrast the bow shock at fixed β and angle between the magnetic field and shock normal but with different Mach number. The second pair show the increase in turbulence associated with an increase in β . The bottom panel shows the case with a very high β . However, the low data rate at this time obscures the detailed structure of the shock.

At Jupiter and Saturn we expect lower β than at 1 AU but higher Mach numbers. This is confirmed in Fig. 2d which shows six jovian bow shocks obtained in only a few days as observed by

the GSFC Voyager magnetometer and their associated plasma parameters. The plasma data necessary for the calculation of Mach number and β were provided by the MIT plasma group. It is evident that the jovian overshoots are much bigger than their terrestrial counterparts. Data for the bow shock of Saturn have recently been published¹¹ and will not be repeated here. The Mach number during the Pioneer 11 passage by Saturn was less than at Jupiter during the Voyager encounters. However, it was still much greater than normally observed at 1 AU.

The two parameters which seem to control the structure of the shock in Fig. 2 are β and Mach number. Thus we have taken a sample of the bow shocks at Venus, Earth, Jupiter and Saturn including those shown and examined the overshoot as a function of β and Mach number. Figure 3 shows the amplitude of these overshoots plotted against β for Venus and the Earth separately and Jupiter and Saturn combined. The scale for Jupiter and Saturn is twice that for Venus and Earth so that there is a different variation at the outer planets. However, qualitatively there is a clear trend of increasing overshoot with increasing β for all the planets.

Figure 4 indicates that the difference between the overshoots at the various planets is due to the difference in Mach number.

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Plotting all the planetary data as a function of magnetosonic Mach number suggests that the trends for each planet coincide. The principal difference being the range of Mach numbers at each planet. Clearly there is also a dependence on β , but the range of β is similar at each planet. We have also checked to see whether the apparent β and Mach number dependences could have been caused by a correlation between β and Mach number but there is, in fact, little correlation between these two parameters, except in the terrestrial data set by itself in which high Mach numbers were always accompanied by high betas.

We conclude that the bow shock structure, notably the magnitude of the overshoot in field strength post-shock, depends on both β and magnetosonic Mach number. This dependence seems to be continuous and we find no evidence for a sudden change in structure at the highest Mach numbers at least as observed in the magnitude of the magnetic overshoot.

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Magnetoid interaction with surrounding stars

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Lugg¹ has recently analysed a magnetoid—a supermassive rotating highly magnetized star²⁻⁵—situated at the centre of a compact galactic nucleus in the belief that previous investigations had taken no account of interaction with neighbouring stars. Such an interaction has been widely discussed⁶⁻¹¹, but there has been no confirmation of the main conclusion of ref. 1 that these stars can disrupt a magnetoid in as short a time as 10^4 yr. Here we demonstrate that this conclusion is erroneous.

On penetrating a magnetoid, stars greatly decelerate and thus, in gradually losing their kinetic energy, settle to the magnetoid's centre. Although this process is accompanied by numerous complex phenomena (such as shock-wave generation, ablation, interaction of radiation with star atmospheres and partial mass loss by stars) we shall assume, as in ref. 1, that a supermassive star (superstar) supported mainly by radiation pressure will be formed eventually from the captured stars at the magnetoid's centre. Lugg believes that such a superstar will collapse as soon as its mass exceeds $10^5 M_\odot$. However, this value for the critical mass used in ref. 1 has been taken from the theory for an isolated superstar whereas in the present case the central superstar is under an appreciable external pressure. We shall show now that in these conditions the critical mass can be much greater than $10^5 M_\odot$.

In the first post-newtonian approximation, the equilibrium energy of a superstar with mass M and radius R experiencing an external pressure $P_s(R)$ on its surface, takes the form

$$\mathcal{E}_e = -(3\gamma - 4)E_{th} + 4\pi R^3 P_s + \int 4\pi r^2 P_s(r) dr + \zeta_1 \frac{G^2 M^3}{R^2 c^2} \quad (1)$$

where $(3\gamma - 4) = \beta/2$, $\beta \equiv (\text{gas pressure}/\text{total pressure}) =$

$4.3 \mu^{-1} (M/M_\odot)^{-1/2}$ where μ is the mean molecular weight per particle. It is convenient to approximate $P_s(R) \sim R^{-q}$ with $q = \text{constant}$. Then, considering the superstar as a polytrope of index three ($\zeta_1 = 5.06$) we have in the critical state ($d\mathcal{E}_e/dR = 0$):

$$R_{cr} = \frac{6.8}{\beta + 2(4 - q)\alpha} R_s \quad (2)$$

$R_s \equiv 2GM/c^2$ being the Schwarzschild radius; $\alpha \equiv P_s/\bar{P} = 94(M_m/M)^2(R/R_m)^4$; M_m and R_m being mass and radius of the magnetoid embracing the superstar. If $M \ll M_m$, then $q \approx 0$ in a good approximation, so that

$$\frac{R_{cr}}{R_s} = \begin{cases} 1.58 \mu (M/M_\odot)^{1/2} & \text{at } \beta \gg 8\alpha \\ 0.85\alpha^{-1} & \text{at } \beta \ll 8\alpha \end{cases} \quad (3)$$

On comparing the upper line in equation (3) (external pressure is negligibly small) with the lower line (external pressure is substantial) it is clear that in the latter case the value of the critical mass, M_{cr} , is much greater. We calculate it in a specific case when the superstar is embedded in a magnetoid of mass $M_m = 10^8 M_\odot$ and radius $R_m = 10^{16}$ cm (the same magnetoid parameters are used in ref. 1). In this case we have $\alpha = 1.3 \times 10^5 (M/M_\odot)^{2/5}$ at $R = R_{cr}$, and the external pressure term is dominating in equation (3) if $M > 1.3 \times 10^5 \mu^{-10/9} M_\odot$. Making R_{cr} equal to the radius, R_b , of the superstar which has reached the hydrogen burning state:

$$\frac{R_b}{R_s} = 6.84 \times 10^5 \left(\frac{M}{M_\odot} \right)^{-0.535} [XZ(1+X)]^{0.069} \quad (4)$$

(ref. 12) we get for the standard population I composition ($X = 0.70$, $Z = 0.03$)

$$M_{cr} = 0.67 \times 10^7 M_\odot \text{ for } M_m = 10^8 M_\odot, R_m = 10^{16} \text{ cm} \quad (5)$$

At this stage, the radius of the superstar of the critical mass $R_b = 1.2 \times 10^2 R_s = 2.4 \times 10^{14}$ cm, that is $R_b/R_m \sim 2 \times 10^{-2}$. Such a small value of this ratio implies that the simplifying assumption $q = 0$ used above to derive equation (3) is well satisfied even for masses as large as M_{cr} given by equation (5).

We see that M_{cr} is much greater than the value $10^5 M_\odot$ used in ref. 1. In other words, the central superstar accumulated from the remnants of captured stars will reach the hydrogen burning

stage even without noticeable influence of external pressure if its mass $M < 2 \times 10^5 M_\odot$ or due to the pressure if $2 \times 10^5 M_\odot \leq M \leq 0.7 \times 10^7 M_\odot$. But in neither case does this superstar collapse. Moreover, the nuclear burning gives the superstar a main sequence lifetime of as long as 10^6 yr, independent of its mass and with only a weak dependence of its initial heavy element content¹² unless the hydrogen abundance of the captured stars is abnormally low.

Lugg¹ has found that the mass accumulated in the central superstar is $M \geq 10^5 M_\odot$, but such a large value is a result of two basic assumptions made in ref. 1. First, it was assumed that the star cluster surrounding a magnetoid has a mass $M_c \ll M_m$. However, there is considerable evidence in favour of an opposite inequality both for active galactic nuclei and for 'quiescent' ones, such as the nucleus of our Galaxy (see below). As a result, the cross-section for interaction of stars with a magnetoid is equal to

$$\sigma = \pi R_m^2 (1 + 2GM_m/R_m v^2) \approx 2\pi R_m r_c (M_m/M_c)$$

that is it is smaller than the value in ref. 1 by a factor M_m/M_c (r_c is the core radius). Furthermore, the value $M_m = 10^8 M_\odot$ used in ref. 1 should be attributed to a magnetoid which has the characteristics of a quasar but not of an active galactic nucleus, and certainly not that of our own Galaxy. From the requirement that the non-thermal luminosity ($\leq 10^{44} - 10^{45}$ erg s⁻¹) observed for active galactic nuclei should be less than or equal to the Eddington luminosity of a hot magnetoid it follows that $M_m \leq 10^6 - 10^7 M_\odot$ in active nuclei. Meanwhile the collision rate of stars with the magnetoid is $f = \sigma n_c v \sim M_m R_m (r_c/M_c)^{1/2} n_c$, n_c being the number density of stars in the core. Taking into consideration that $R_m \sim M_m$ (ref. 5) we have $f \sim M_m^2$, that is f decreases greatly as M_m diminishes.

A comparatively low value of M_m is expected in active galactic nuclei, making it reasonable for an inequality $M_m \ll M_c$ (opposite to that used in ref. 1) to take place there. This agrees well with the fact that the velocity dispersion of stars in the cores of both M31 and our Galaxy is as low as $v \approx 200$ km s⁻¹ being determined by the gravitational field of an extended mass $M_c \sim 10^7 - 10^8 M_\odot$, and not by that of a point (magnetoid) mass of value $M_m = 10^8 M_\odot$ which gives¹ $v \approx 600$ km s⁻¹. Therefore, if the activity of a Seyfert nucleus is conditioned by the central magnetoid acting within a normal stellar core, inequality $M_m \ll M_c$ will be satisfied there. This inequality will only be stronger if the Seyfert phenomenon originates in a galaxy having a more powerful spherical component. (Note that there is strong evidence for such an inequality within the framework of black hole models; see refs 13, 14).

The lower value of M_m and inequality $M_m \ll M_c$ in active nuclei both suggest that the total mass of stars which succeed in sticking inside the magnetoid in the time of refilling of low angular momentum orbits should be considerably less than the value $\geq 10^5 M_\odot$ of ref. 1. Even if this mass were much greater (for example, because of collective gravitational effects) but less than M_c given above, it would lead not to its collapse but to a rather slow evolution on the nuclear time scale of about 10^6 yr—comparable with the lifetime of an isolated magnetoid. Therefore, the conclusion in ref. 1 about the rapid disruption of a magnetoid by the surrounding stars is wrong.

Nevertheless, interaction of stars with the magnetoid has several important and interesting aspects (especially from an observational point of view) mentioned in refs 6–11. Note that we do not regard a magnetoid as the only possible 'engine' responsible for the activity of quasars and nuclei of galaxies. The advantages and difficulties of three possible sources of activity—a massive black hole, a magnetoid and compact star cluster—are discussed in detail elsewhere^{15–18}. The observational tests proposed in refs 15–18 would help to create a realistic theory of the engine.

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Initial microbiological response in lakes to the Mt St Helens eruption

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No published reports exist on the early stages in the establishment and succession of microbial communities and associated chemical and geochemical transformations in aquatic environments shortly after a volcanic event. Much of our understanding of volcanic lakes has come from studies carried out years to centuries after the eruption, when the lakes were already stabilized. Here we document the chemical and microbiological responses of lakes and hydrothermal environments in the blast zone near Mt St Helens 3–4 months after the eruption of 18 May 1980. Within weeks of the eruption, the impacted lakes became anaerobic, ultraeutrophic and developed extensive populations of bacteria, some of which were not common to those environments. By August 1980 both assimilative and dissimilative nitrogen cycle reactions were regulating the extent of chemosynthetic, photosynthetic and heterotrophic activities occurring in the lakes. Recovery of the lakes to their normal oligotrophic state will result in part from oxygen production by photosynthesis and anaerobic oxidation of reduced metals, gases and organic compounds coupled with denitrification.

There are numerous oligotrophic sub-alpine lakes surrounding Mt St Helens (Fig. 1), which were dramatically altered by the 18 May 1980 eruption. Some lakes and streams were reshaped, new lakes and numerous hydrothermal seeps emerged. Aquatic environments in the blast zone received massive quantities of ash, soil, wood debris and pyrolyzed soluble organics from the destroyed forest. Previously oligotrophic lakes became ultraeutrophic and anoxic.

The differences in the chemical and biological characteristics of the Mt St Helens lakes in August and September 1980 compared with before the 18 May eruption generally reflected the degree of impact. The lakes can be grouped into three categories based on impact: (1) those receiving only ashfall, such as McBride, June and Merrill; (2) lakes in the blowdown or scorch zones, such as Ryan, Boot, Meta, Panhandle, Venus, Island, Hanaford, Fawn and St Helens; and (3) lakes affected by mud and debris flows, including Spirit, North and South Coldwater, Castle Creek and other new lakes formed by mudflows damming the Toutle River. The heavily impacted lakes near the crater and in the path of mudflows soon became anaerobic due to increased microbial activity fuelled by the high concentrations of dissolved organic carbon (DOC), sulphur compounds and metals entering the lakes, with the result that massive levels of reduced metals, gases and low molecular

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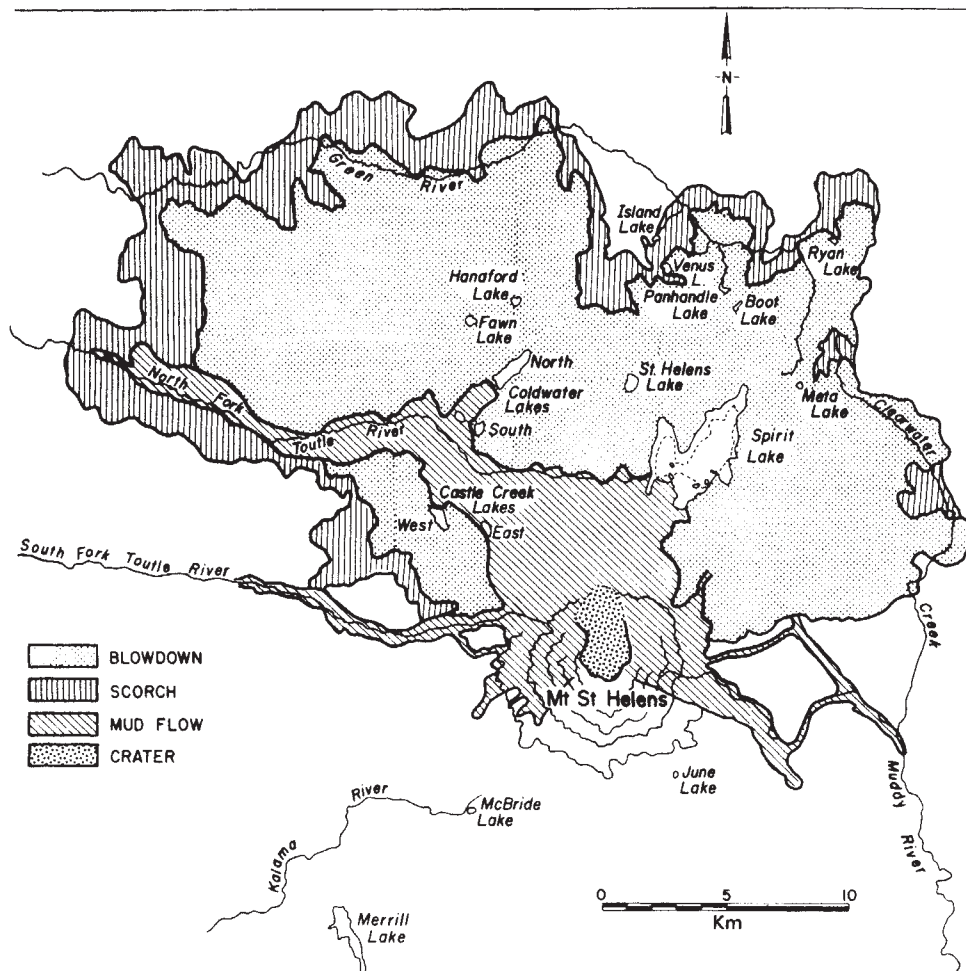


Fig. 1 Map of the lakes in the region surrounding Mt St Helens, showing areas of blowdown, scorch and mudflows. The lakes outside these zones received ashfall but were not in the blast zone.

weight organic compounds were mobilized into the water column. Lakes in the blowdown or scorch zones of the blast area maintained aerobic surface layers over anaerobic hypolimnia. A thermocline was present in lakes in blowdown and scorch zones. Lakes outside the blast zone, which received only ash, have changed the least and remain oxygenated.

All lakes near the volcano have received periodic input of volcanic ash, of dacitic composition, consisting primarily of silica

and aluminium oxides^{1,2}, and mainly of sand size³. Elements of potential biological significance in the ash include Fe (3.7%), P (0.10%), Mn (0.07%) and Mo (1.9 p.p.m.). Leaching experiments using a water/ash ratio of 10:1 in aerobic conditions yielded no decrease in pH and released into solution the following nutrients (in p.p.m.): SO_4^{2-} (57), PO_4^{3-} (3), Mn (0.4), Fe (0.01) and Mo (>0.005)^{1,2}. Most of the constituents of the ash are insoluble in aerobic conditions.

Table 1 Physical and chemical characteristics of lakes and hydrothermal seep in the blast zone of Mt St Helens

Measurement	Ryan (surface)	Ryan (3m)	Ryan (6m)	Lakes Spirit (surface)*	N. Coldwater (surface)	N. Coldwater (seep)	McBride (surface)
Distance from crater (km)	19	19	19	5	11	11	9
Depth (m)	6	6	6	15	34	0.2	7
Area (ha)	1.6	1.6	1.6	700	100	1-3m ²	3.6
Temperature (°C)	20	14	7	19	22	72-80	16
pH	6.88	6.55	6.43	6.80	6.21	5.35	7.26
O ₂ (mg l ⁻¹)	1.43	0.00	0.00	0.00	1.61	0.00	8.72
ΣCO ₂ (mM)	0.25	0.55	1.58	2.05	3.60	ND	0.14
Fe ²⁺ (mg l ⁻¹)	0.18	1.21	2.57	1.08	6.27	9.26	0.00
Mn ²⁺ (mg l ⁻¹)	0.71	ND	ND	4.48	3.35	ND	ND
PO ₄ ³⁻ P (mg l ⁻¹)	0.014	0.020	0.021	0.031	0.022	0.790	ND
Total P (mg l ⁻¹)	0.027	0.045	0.027	0.042	0.043	1.556	ND
NO ₂ ⁻ + NO ₃ ⁻ (mg l ⁻¹)	0.010	0.000	0.000	0.000	0.000	0.020	0.011
NH ₄ ⁺ (mg l ⁻¹)	0.012	0.009	0.031	0.039	0.000	0.316	ND
DON (mg l ⁻¹)	0.195	0.087	0.123	0.213	0.690	4.204	ND
DOC (mg l ⁻¹)	10.2	16.3	46.4	51.1	114	4,285	3.7
DOC/DON	52	187	377	240	165	1,019	ND
N ₂ O (nM)	8.8	7.0	0.6	7.4	6.1	18.8	9.4
H ₂ (nM)	4.1	5.0	1.0	2.0	20->200	15.5	9.0
CO (nM)	238	2.58	87	193	100->150	395	54
CH ₄ (μM)	5.2	8.2	12.3	15.4	28->250	1-6.3	1.3

Data are from sampling on 10 August, 19 August, or 11 September 1980. The methods used for chemical measurements are reported in the references given in parentheses: O₂ (16); CO₂ (17); Fe²⁺ (18); Mn (19); PO₄P, NH₄⁺, NO₂⁻, NO₃⁻ and DON (20); total P (21); DOC (22); N₂O (23); and H₂, CO and CH₄ (using a modification of the procedures in ref. 24). ND, no data.

* Surface water temperature at Spirit Lake on 19 May 1980, the day after the major eruption, was 34 °C (T. Casadevall, personal communication). Surface temperature measured on 4 April 1980 was 4 °C (R. C. Wissmar, personal communication). Historical data indicate that typical temperatures for the middle of May would be ~10 °C. The surface concentration of DOC on 4 April 1980 was 0.83 mg C l⁻¹ in Spirit Lake (R. C. Wissmar, unpublished data). Surface values for DOC on 30 June, 11 August and 19 August 1980 were 39.9, 39.9 and 51.1 mg C l⁻¹ respectively.

Table 2 Microbiological characteristics of lakes and hydrothermal seep in the blast zone of Mt St Helens

Measurement	Ryan (littoral)	Ryan (midsurface)	Ryan (3m)	Ryan (6m)	Lakes Spirit (surface)	N. Coldwater (surface)	N. Coldwater (stream)	N. Coldwater (isolated pond)	N. Coldwater (hot water seep)
Total no. bacteria per ml	3.0×10^8	3.0×10^7	1.5×10^7	6.4×10^6	4.2×10^8	$3.4-7.0 \times 10^7$	1.2×10^8	$4-6.7 \times 10^7$	9.1×10^6
Total no. sulphur/Mn- oxidizing bacteria per ml	$0.8-1.0 \times 10^6$	5.0×10^4	2.0×10^6	2.0×10^6	5.0×10^6	$3-3.4 \times 10^5$	3.0×10^6	$2-5.5 \times 10^2$	ND
N ₂ fixation (light aerobic nM N ₂)	210 (cm ⁻² h ⁻¹)	667 (l ⁻¹ h ⁻¹)	ND	ND	ND	ND	ND	NDet	NDet
N ₂ fixation (dark anaerobic nM N ₂ l ⁻¹ h ⁻¹)	140	130	180	220	400	180->230	170	NDet	NDet
Denitrification (μM N ₂ O l ⁻¹ day ⁻¹)	<1	<1	10	30	36	50	70	12	5
Nitrification (μg NO ₂ l ⁻¹ day ⁻¹)	12	2	<0.1	<0.1	<0.1	<0.1	0.4-3.0	<0.1	<0.1
Presence/absence of: cyanobacteria	+	+	-	-	+	-	ND	-	-
algae + diatoms	+	+	-	-	+	+	ND	-	-

Data are from sampling on 10 August, 19 August, or 11 September 1980. Total bacterial counts were determined using epifluorescent microscopy²⁵. Manganese- and sulphur-oxidizing bacteria were counted using a medium of the following composition (in g): NaCl, 2.6; KCl, 0.88; MgCl₂, 0.56; MgSO₄, 0.76; CaCl₂, 0.1; Na₂HPO₄, 9; KH₂PO₄, 1.5; NaNO₃, 1.0; Na₂S₂O₃, 5; FeSO₄·7H₂O, 20×10^{-3} ; MnSO₄·H₂O, 150×10^{-3} ; Bacto yeast extract (Difco), 0.01; trace element solution according to ref. 26, distilled water to 1 l and adjusted to pH 6.8. Bacto purified agar was dialysed in distilled water to remove soluble organics and 12 g l⁻¹ used. Manganese oxidation was measured using a benzidine reagent as described in ref. 27. The procedures used for microbiological activity measurement were: N₂ fixation, ref. 28; denitrification, ref. 29; nitrification, ref. 30. The rates of N₂ fixation are only apparent. The acetylene reduction method is an indirect assay for fixation, and the rates have not yet been verified with ¹⁵N₂. Conversion to nanomoles of N₂ fixed was done using an assumed theoretical ratio of 3.0 mol of ethylene produced per mol of N₂ fixed. The theoretical ratio has been reported to vary from 2.0 to 25 (ref. 31). Recently a conversion factor for an anaerobic bacterial population, *Microcoleus chthonoplastes*, in solids was given³² as 5.4. Although the rate of N₂ fixation may be about double the actual rate, it is still much greater than previously reported anaerobic dark fixation rates in a water column. Blue-green algae isolated from lakes in the blast zone included *Pseudanabaena*, *Anabaena* and *Oscillatoria*. Other algae found included *Nitzschia*, *Euglena*, *Spirogyra* and an encysting motile, unicellular green alga. ND, no data; NDet, not detected.

In the blast zone, the tremendous heat associated with the eruption (200–350 °C) pyrolyzed much of the coniferous forest foliage. Throughout the mudflow, blowdown and scorch zones, the concentration of DOC and particulate organic carbon (POC) increased greatly, and the temperature was higher than in nearby lakes outside the blast zone (Table 1). DOC in Spirit Lake increased from <1.00 mg Cl⁻¹ to >30 mg Cl⁻¹, and in North Coldwater and Castle Creek Lakes DOC exceeded 100 and 200 respectively. Heterotrophic microbial activity coupled with the decreased solubility of oxygen at the elevated temperatures quickly consumed the available O₂. On 30 June 1980, in Spirit Lake, dissolved O₂ was 2.35 mg l⁻¹ at 0.25 m (R. C. Wissmar, unpublished data). Throughout August and September no O₂ was detected at 0.2 m in Spirit, Castle Creek and North Coldwater Lakes. Lakes outside the path of mudflows but in the blast zone were anaerobic at depth but retained a surface layer with some dissolved O₂; those receiving only ashfall were oxygenated throughout.

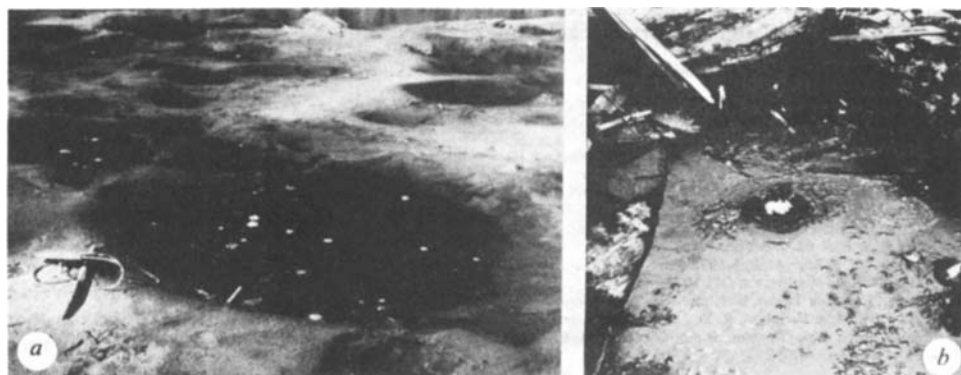
During the summer and early autumn of 1980, we made four 1-day expeditions to lakes and hydrothermal seeps in the blast zone of Mt St Helens. Ryan, Spirit and North Coldwater Lakes, and the hydrothermal seeps near North Coldwater, were sampled most intensively. The water at all sites was light brown to black in colour due to high levels of sulphides and DOC (Fig. 2). The sediments were actively degassing, giving off methane and other gases, so that in some lakes, such as North Coldwater, the entire surface was continuously bubbling (see Fig. 2). The

bubbles in the lake and at isolated puddles at North Coldwater were collected and analysed and found to consist of ~50% CH₄, 50% CO₂ and trace levels of H₂, CO and N₂O. At the hydrothermal seeps, a black odoriferous liquid with temperatures up to 80 °C was emerging and multi-coloured organic films (oil slicks) formed on the surface. The films were periodically broken apart due to violent degassing of, primarily, CH₄, CO₂ and CO (Table 1).

The threefold increase in carbon, phosphorus, sulphur, iron and manganese concentrations, the presence of dissolved gases and the microbial activities in the water suggest that nitrogen cycle processes, including nitrogen fixation, nitrification and denitrification, are important (Tables 1, 2). Dissolved organic nitrogen (DON) concentrations remained low, due to the low C/N ratio of the conifer remains blasted into the lakes and the paucity of nitrogen in volcanic ash^{4,5}, and resulted in low inorganic nitrogen (NH₄⁺, NO₂⁻, NO₃⁻) concentrations, high rates of nitrogen fixation and high DOC/DON ratios. For example, Ryan Lake, which maintained an oxygenated surface layer and algal flora, had a C/N ratio of 52 at the surface but 377 near the bottom. Spirit and North Coldwater Lakes had ratios of 150–250 while that at the hydrothermal seeps exceeded 1,000. The nitrogen concentrations, relative to carbon, phosphorus and sulphur, suggested a nitrogen limitation.

Such a notion was supported by the high rates of phototrophic N₂ fixation and anaerobic dark N₂ fixation (Table 2). Anaerobic dark N₂ fixation was ≤230 nM N₂ l⁻¹ h⁻¹ in the surface waters

Fig. 2 Photographs of *a*, isolated pond near North Coldwater Lake, ~1 week after being filled by rain and showing extensive outgassing and dark black coloration of the water (pond diameter is ~1.5 m) and *b*, 80 °C hydrothermal seep near North Coldwater creek. Surface of the seep is covered over by a dark, 1–2 cm dark organic sulphide crust which looks like an oil slick.



of North Coldwater Lake. Brezonik and Harper reported a maximum rate of $11 \text{ nM l}^{-1} \text{ h}^{-1}$ in one of three lakes with extensive anoxic environments⁶. Such high rates of fixation have not previously been reported in these conditions, and represent a major source of reduced nitrogen to the lakes in the blast zone of Mt St Helens.

The number of bacteria in the lakes and seeps in the blast zone routinely exceeded 10^8 organisms per ml (Table 2). The manganese/sulphur oxidizing bacteria were repeatedly detected in excess of 10^6 per ml, a value comparable with the total bacterial counts in eutrophic lakes which generally range from 10^6 to 10^7 organisms per ml (ref. 7). Very active methanogenesis occurred in the anaerobic sediments of all lakes and hydrothermal seeps. In addition to ebullition of CH_4 -rich bubbles at all sites, the dissolved levels ranged from 5 to $>250 \mu\text{M}$. High levels of CO , H_2 and N_2O were also detected in all impacted lake waters (Table 1). The exceedingly high concentrations of CO in lake surface waters at ambient temperature and North Coldwater hydrothermal seep waters have not been previously reported. The microbiological processes involved in CO production are not well understood.

Denitrifying bacteria were present in all but the oxygenated surface waters at Ryan Lake, even at 78°C at the North Coldwater seep. The manganese/sulphur oxidizing bacteria were the most abundant denitrifying bacteria, but unlike most stratified and meromictic lakes, where the metal-oxidizing bacteria are detected only at the oxycline, in Mt St Helens lakes the denitrifying metal oxidizers oxidize throughout the anaerobic water column and thus resemble estuarine sediment denitrifiers. Whereas denitrification was occurring in all aquatic environments except the surface waters at Ryan Lake, significant nitrification was limited to the oxygenated zone at Ryan Lake with greatest activity detected in the littoral zone, apparently associated with the nitrogen-fixing cyanobacteria. Significant rates of nitrification were detected in the oxygenated stream that runs into North Coldwater Lake and it is presumed that the stream is the source of nitrate for the lake (Table 2). This interpretation is supported by the reduced rates of denitrification in the isolated pond at North Coldwater.

Overall, the numbers and activities of the bacterial community reflected the widespread anaerobic conditions in the lakes and hydrothermal seeps in the blast zone in August and September 1980. The rates of this microbial biogeochemical cycling in the waters in the blast zone near Mt St Helens rivals those normally found in estuarine muds or the sediments of eutrophic lakes⁸⁻¹⁵. We believe that the rate at which these ecosystems return to the oligotrophism existing before the 18 May eruption will be largely regulated by nitrogen cycle processes associated with bacterial and algal metabolism.

Finally, we believe that many of the heavily impacted lakes and hydrothermal seeps on Mt St Helens represent ideal experimental sites for studying anaerobic microbial processes and chemical transformations which closely resemble those suggested to characterize Archaea reducing aquatic environments.

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Speculated cause of interhemispheric oceanic oscillation

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The study of large-scale correlations of atmospheric and oceanic variables on a supra-annual scale is essential for an understanding of climatic variability. We present here new data on the mean sea-level anomalies in the eastern Indian Ocean which are consistent with supra-annual anomalies in the intensity of the main oceanic gyre, and are negatively correlated with mean sea-level anomalies in the eastern Pacific Ocean. Larger anomalies are induced in the Indian Ocean probably due to its smaller size, and zonal mean stratospheric 50 mbar geopotential heights at 10°N , 20°N and 30°N are better correlated with the Indian Ocean than with the Pacific Ocean mean sea levels. It is speculated that the reported responses are due to a random variation of the monsoonal generated heat transport between the oceans. This variability would give rise to equatorial heat anomalies in each ocean which induce the observed changes in mean sea level, and the coupled changes in the sub-tropical atmospheric circulations.

Figure 1 shows the annual mean sea levels in the Indian Ocean, based on new data for Australian ports¹. A strong coherence clearly exists between the port groups and a lag correlation analysis (J. S. Godfrey, personal communication) indicates that the level changes appear almost simultaneously (within 2 months) throughout. This coherence is similar to that occurring in the eastern Pacific Ocean between San Francisco (38°N , 122°W) and Matarani (17°S , 72°W)², however, a clear tendency for poleward propagation of the level changes has not been established for the Indian Ocean data. Furthermore on comparing the South Australian port group (for which earlier data are also available) with a group of California ports for the period 1944-75 (Fig. 2), a remarkable negative correlation (correlation coefficient -0.69) between the two records is apparent. Typically each record shows annual mean sea-level variations of ± 5 cm on a period of 3-7 yr. A spectral analysis of each of the records gives multiple peaks of energy in this band.

These data suggest that the mean sea-level variations are due to large-scale phenomena in each ocean, linked by a common mechanism. We propose that the level changes can be interpreted as a strengthening or weakening of the major oceanic sub-tropical anticyclonic gyres, such that for a more intense gyre the anomaly of levels would be positive at the centre, and negative around the coasts, and vice versa. We develop this idea quantitatively using simple models of large-scale ocean circulation^{3,4}. We also speculate on the mechanism that initiates and

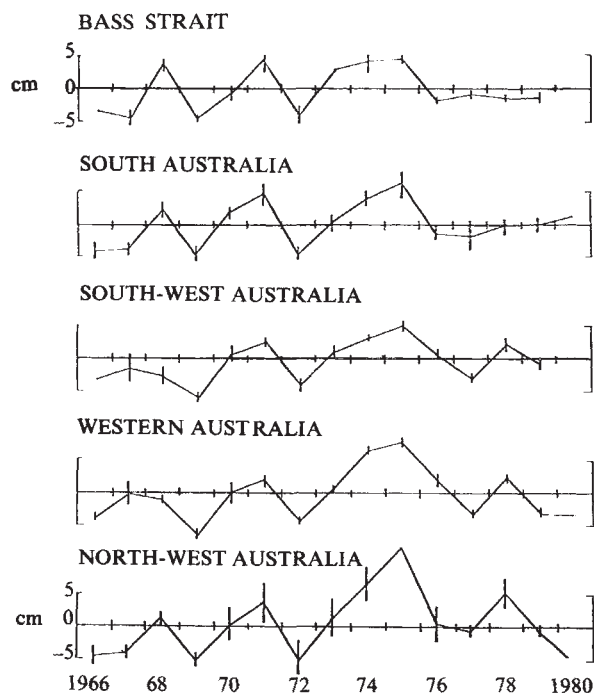


Fig. 1 Deviations in annual mean sea levels in the eastern Indian Ocean for the period 1966–80. Port groups: Bass Strait (Point Lonsdale (38°S, 145°E) and Georgetown (41°S, 147°E)); South Australia (Thevenard (32°S, 136°E), Port Lincoln (35°S, 136°E), Port Macdonnell (38°S, 141°E)); south-west Australia (Albany (35°S, 118°E) and Esperance (34°S, 122°E)); Western Australia (Geraldton (29°S, 115°E), Fremantle (32°S, 116°E) and Bunbury (33°S, 116°E)); north-west Australia (Darwin (12°S, 131°E), Port Hedland (20°S, 119°E) and Carnarvon (25°S, 114°E)). The bars indicate standard deviations between ports; a zero standard deviation is shown when data from only one port are available.

maintains the negatively correlated anomalies in each ocean. We consider first a mechanism for establishing the anomalies.

The Pacific Ocean and the Indian Ocean are connected to the north through the Indonesian archipelago, and to the south by the Southern Ocean. The northern connection lies along the track of the South-East Asian monsoon⁵, which essentially blows towards the north in the northern summer and towards the south in the northern winter, and drives a corresponding circulation in the surface waters of the passages in the archipelago. If there were a random variation in the strength of the monsoon, this would be reflected in a random variation of the heat transport between the two oceans. We suggest that this exchange of heat is the prime cause of the responses shown in Fig. 2, because a positive flux anomaly for one ocean is a negative flux anomaly for the other ocean, and vice versa. Hence the supra-annual anomalies in heat content in each ocean would be negatively correlated, their magnitudes being functions of the monsoonal variability of the previous few seasons. This conclusion is an example of the Slutsky effect⁶ whereby a moving average of a series of random numbers tends to give rise to a series which is quasi-periodic at a period approximately twice that of the length of the moving average.

The differences between the observed records are due to factors in each ocean that effectively give rise to dissimilar moving averages. Notably, the monsoonal heat flux after entering the Indian Ocean is carried westward by the south equatorial current, whereas in the Pacific Ocean its advent is opposed by the north equatorial current with the result that there is a tendency for a ponding of heat anomaly in the western Pacific Ocean. The dispersion of this pond is achieved principally through the equatorial undercurrent, and at times by a sudden discharge which results in an El Niño event^{7–9}. The Indian Ocean mean sea-level regime indeed extends into the western Pacific Ocean at least as far as Truk (7°N, 151°E)²¹. Note also that although the monsoonal heat exchange for geographical

reasons is primarily an interhemispheric exchange between the southern Indian Ocean and the northern Pacific Ocean, the effect of heat anomalies along the Equator is transmitted to the general circulations of both hemispheres.

In a baroclinic ocean the anomalies in heat content are reflected in surface level changes through the presence of a sub-surface reference level which remains approximately isobaric during the variability. Thus we hypothesize that monsoonal generated changes in ocean heat content in the equatorial regions are the primary mechanism for the induction of the observed anomalies of mean sea level. In particular, in the equatorial regions the depth of the reference level is ~300 m (ref. 10), from which it is deduced that temperature anomalies of $\pm 1^\circ\text{C}$ would induce mean sea-level anomalies of $\pm 5\text{ cm}$, an increase in level corresponding to an increase in temperature.

Consider next the implications of the persistence of the equatorial anomalies for the general circulations in the ocean and the atmosphere. First, the vorticity balance in the oceanic anticyclonic gyres requires that a cold anomaly (a negative mean sea-level anomaly) must be balanced by an increase in the anticyclonic wind stress curl. In fact, for an ocean gyre in a rectangular basin (latitudinal width L , meridional width M), which is driven by an anomalous zonal wind stress distribution, $\tau = -\tau_0 \cos \pi y/M$, an approximate expression¹¹ for the anomaly of sea level on the perimeter of the basin, except along its western boundary, (assuming no change in the mass of water in the basin) is

$$\eta \sim -\frac{\tau_0}{\rho g H} \frac{f}{\beta} \frac{L}{M}$$

where ρ is the density of seawater, g is the acceleration due to gravity, $f = 2\Omega \sin \phi$ is the Coriolis parameter in which Ω is angular speed of rotation of the Earth, and ϕ is latitude, $\beta = df/dy$ ($0y$ towards the north), and H is the depth of the reference level. Thus, assuming $L = 10,000\text{ km}$, $M = 3,000\text{ km}$, $\rho = 1,000\text{ kg m}^{-3}$, $g = 10\text{ m s}^{-2}$, $H = 300\text{ m}$, $|f| = 0.3 \times 10^{-4}\text{ s}^{-1}$, $\beta = 2 \times 10^{-11}\text{ m}^{-1}\text{ s}^{-1}$, a negative anomaly of mean sea level of 5 cm would induce an increase in the stress contrast between the trade winds and the westerlies $|2\tau_0|$ of 0.05 N m^{-2} . This value is of the order of 25% of the annual mean contrast of 0.2 N m^{-2} . It is significant that the only latitudinal variation in the (geostrophic) expression for η lies with f and H , and thus η is predicted to be of similar form at all latitudes in agreement with the data (Fig. 1). The observed similar magnitudes of the anomalies at all latitudes suggest that the reference level $H \propto f$, an inference consistent with oceanic thermocline theory¹². As L is smaller in the Indian Ocean than in the Pacific Ocean, and η is probably larger (Fig. 2), the expression for η indicates that the induced changes in τ would be more significant in the Indian Ocean than in the Pacific Ocean.

In addition to the redistribution of mass within the ocean basins due to the gyral circulation, there is an exchange of mass between the ocean basins brought about through the inverse barometer effect¹³ due to changes in atmospheric pressure. These level changes (ζ) are quite well correlated within the Indian Ocean (Table 1). The ratio of the standard deviations

Table 1 Standard deviations of annual mean sea level and atmospheric pressure in the Indian Ocean for the period 1966–80

Port Group	$(\bar{\eta}^2)^{1/2}$ (mm)	Atmospheric pressure station	$(\bar{\zeta}^2)^{1/2}$ (mm)	Correlation coefficient between η and ζ
North-west Australia	48	Darwin	6	0.77
Western Australia	38	Perth	8	0.54
South Australia	33	Adelaide	12	0.93

$\zeta = -P_a/\rho g$ is the anomaly of mean sea level arising from the inverse barometer effect, η is the observed anomaly of mean sea level.

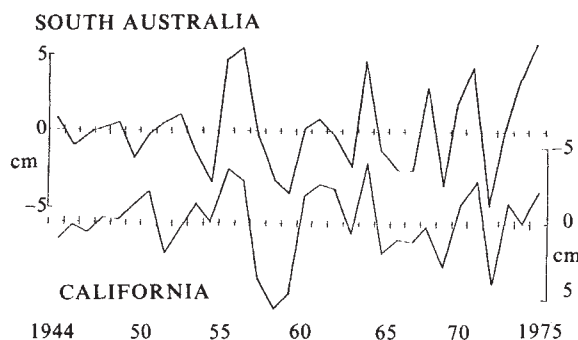


Fig. 2 Deviations in annual mean sea levels on the coasts of South Australia and California for the period 1944–75. Port groups: South Australia (Thevenard, Port Lincoln and Port Macdonnell (1959–75) and Port Adelaide (Outer Harbor 35°S, 138°E, 1944–70); California (San Francisco (35°N, 122°W), Los Angeles (34°N, 118°W), La Jolla (33°N, 117°W) and San Diego (33°N, 117°W)). The records for each California port²¹, and for the South Australian port group^{1,21} were detrended before the calculation of the deviations. Note the scale for the California port group is inverted.

$(\zeta^2/\eta^2)^{1/2}$ gradually increases from north to south, but even for South Australia, the inverse barometer effect accounts for only a small proportion of the variability in mean sea level.

Assuming that the anomaly of atmospheric pressure has the form, $P_a = P_0 \sin \pi y/M$, and applying the geostrophic relation, $\rho_a u_a = -\partial P_a / \partial y$ in which ρ_a is the surface density of air, and u_a is the anomaly of the surface geostrophic wind, it can be easily shown that the anomalous difference in velocity between the trade winds and the westerlies is $|2\pi P_0 / M f \rho_a|$. Thus, substituting $P_0 = 1$ mbar (Table 1), $\rho_a = 1.2 \text{ kg m}^{-3}$, and $|f| = 10^{-4} \text{ s}^{-1}$, this velocity difference is 2 m s^{-1} , which, assuming a surface drag coefficient of 1.3×10^{-3} (ref. 14) is equivalent to an anomalous stress contrast between the trade winds and the westerlies of 0.06 N m^{-2} , in excellent agreement with the calculation based on the gyral circulation.

Note that the supra-annual behaviour of the atmospheric general circulation in the Indian Ocean was described in 1925 by Kidson¹⁵ using data for the period 1890–1923. Kidson selected a period of 36 months which he called 'a natural period of action and reaction' as being representative of the major variability. This period is somewhat less than periods (36–60 months) usually adduced from Pacific Ocean data in later studies¹⁶. The two data sets, however, may be reconciled through the discussion of the interaction between the two oceans given here.

We now present new data on tropical stratospheric geopotential heights¹⁷. The zonal annual mean anomalies for 10°N, 20°N and 30°N are all greater in magnitude than the surface pressure anomalies (Table 1), and are well correlated with the mean sea-level data (Table 2). The magnitudes of the correlation coefficients are higher with the South Australian than with the California mean sea-level data, the former being negative and the latter positive. It seems therefore that the zonal mean stratospheric pressure anomalies are in phase with the tropospheric pressure anomalies in the Indian Ocean, rather than linked barotropically with the Pacific Ocean pressure anomalies.

Table 2 Standard deviations of zonal annual mean geopotential heights at 50 mbar, and 10°N, 20°N and 30°N, for the period 1958–75, and the annual mean sea level for California and South Australian ports

Latitude (°N)	Geopotential height (η^2) ^{1/2} (gpm)	Mean sea level		Correlation coefficients between p and η p and η'	
		California ports (η^2) ^{1/2} (mm)	South Australian ports (η^2) ^{1/2} (mm)		
10	18			0.33	–0.55
20	17	24	32	0.43	–0.58
30	15			0.48	–0.64

An increase of 1 gpm at the sea surface would lower sea level, by the inverse barometer effect, 1 mm.

This remarkable observation is consistent with an earlier conclusion that the changes in the atmospheric general circulation would be more significant in the Indian Ocean than in the Pacific Ocean. Thus the Indian Ocean climatic signature would be more likely to be impressed on the zonal stratospheric data. Gordon¹⁸ has already reported a highly significant period of about 37 months from a spectral analysis of the monthly anomalies of the stratospheric data, suggestive of Kidson's conclusions for the surface Indian Ocean data. The period possesses greatest power between longitudes 120° and 180°E. Joseph¹⁹ has also found an approximate triennial oscillation in the wind at 100 mbar in the equatorial regions of Asia and Africa. In the peak westerly phase of the oscillation the subtropical westerly wind belt of the Northern Hemisphere penetrated southwards as far as or even beyond the Equator. He found that these incursions appeared to coincide with the failure of the Indian summer monsoon.

We have discussed evidence for a coupled long period inter-hemispheric oscillation of the ocean and the atmospheric, but the fundamental question is what maintains the oscillation. It may be argued that the ocean is simply responding to the atmospheric southern oscillation which has a similar variability to that deduced from the oceanographic data²⁰. We suggest, however, that the oceanic anomalies are induced by the variability of the monsoonally generated oceanic heat flux, the effect of which is to modulate the vorticity balance of the sub-tropical oceanic gyres. This argument has the important corollary that the subtropical atmospheric circulation must also change sympathetically to support the changes of ocean vorticity.

Thus our proposed mechanism implies that the ocean is the active partner in the supra-annual variability discussed which is characterized in the ocean by an interhemispheric oscillation, and in the atmosphere by the southern oscillation.

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Phanerozoic land-sea and albedo variations as climate controls

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It has been suggested^{1–8} that variations in the latitudinal distribution of land and sea have been important in controlling climate, especially the initiation of glacial epochs, through geological time. It is thought that surface albedo may be increased and ice caps initiated by the placement of land (and hence snow cover) near the poles^{2,6} or by the placement of land

in low latitudes^{4,5,7} or the placement of land with extensive deserts in low latitudes⁸. I document here the relevant Phanerozoic land-sea variations based on recent global palaeogeographical reconstructions⁹, to assess total surface albedo variation and use these data to test the above mentioned hypotheses.

Palaeocontinental reconstructions for the Cambrian to the Triassic (570–200 Myr) have been attempted⁹ using all reliable palaeomagnetic data available in May 1981. These maps (Fig. 1) were constructed using the smallest subdivisions of geological time allowed by the data (epochs, early, medial, late Cambrian and so on). As usual with such palaeomagnetically defined reconstructions, longitudinal placement is often uncertain (especially during the early Palaeozoic) whilst latitudinal position is usually constrained to within $\pm 10^\circ$, though for several blocks the precision may be much better. Fortunately, palaeoclimatic hypotheses are based almost solely on palaeolatitude. The palaeolatitudinal positions of certain continental blocks (North and South China, South-East Asia, Kolymia¹⁰) are uncertain for much of the Phanerozoic and their positions have been plotted by interpolating between and extrapolating beyond the currently available palaeomagnetic data. The distribution of

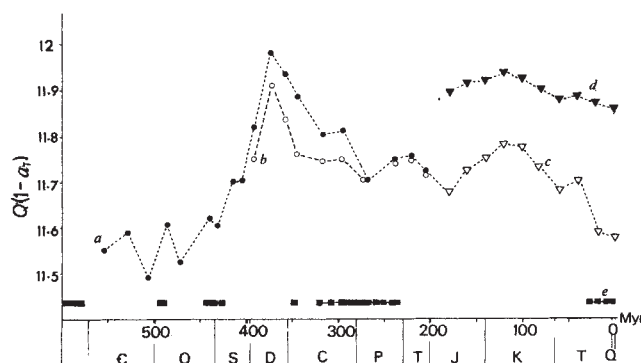


Fig. 2 Variation of $Q(1-a_T)$ (solar radiation absorption) at Earth's surface through the Phanerozoic. See ref. 8 for details of calculation. *a*, $\bullet$, $Q(1-a_T)$ values assuming no deserts or, for Permo-Trias, only those areas known from geological evidence to have been desert from the geological record; *b*, $\circ$, values with desert prescribed 10° – 30° ; *c*, Δ , post-Triassic trend from ref. 8 assuming no deserts; *d*, $\blacktriangle$, post-Triassic trend from Barron *et al.*⁸ assuming deserts 10° – 30° ; *e*, major Phanerozoic ice ages.

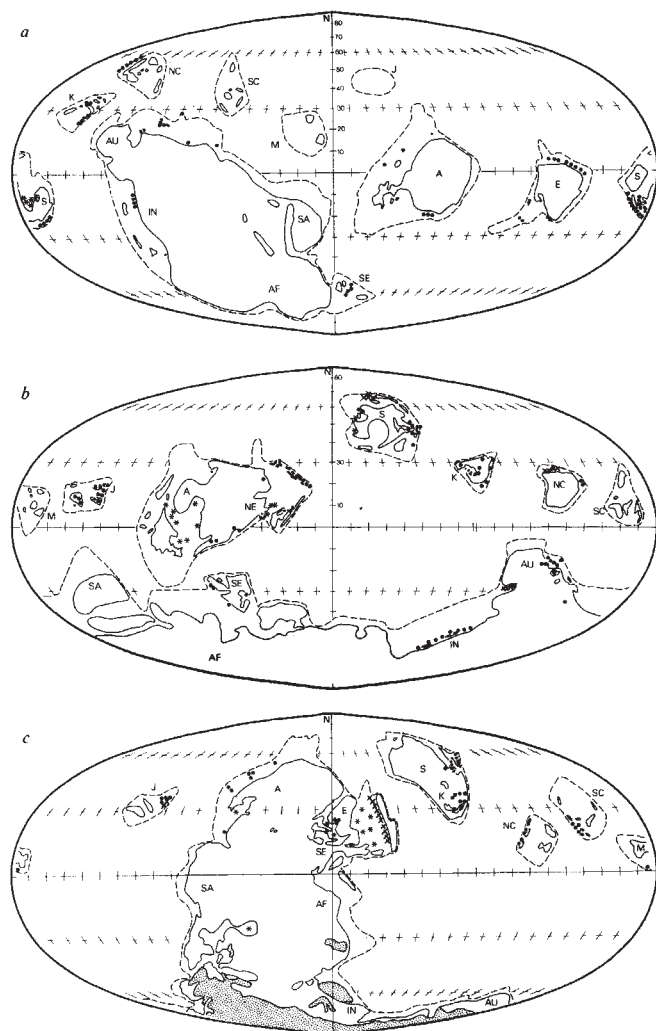


Fig. 1 Palaeogeographical maps for: *a*, late Cambrian; *b*, early Devonian; *c*, early Permian. Solid lines indicate palaeoshorelines; $\bullet$, major volcanics; $\star$, evaporites; $\times$, coral reefs. Major ice-caps are shown by stipple. The plot is a combined Lambert cylindrical and sinusoidal equal-area projection. The extent of continental blocks is defined elsewhere¹⁰. A, North American block (including Greenland); AF, Africa; AU, Australia; IN, India (including Tibet); J, Jano-Kolymian block; K, Kazakhstan block; M, South-East Asian block; N, Palaeo North Pole; NC, North China block; NE, Northern European block; S, Siberian block; SC, South China block; SE, Southern European block.

Palaeozoic and Triassic epicontinental seas was then plotted using over 3,000 recent references.

The distribution of epicontinental seas is critical in consideration of past albedo as these seas have often almost completely inundated certain large continental blocks and they cannot be ignored as was done by Cogley^{7,11}. Palaeogeographical atlases^{12,13} based on outmoded data and preconceptions, although still used by some^{8,14,15} have not been used here. A preliminary approximation of major land surface types (deserts, forests and so on) has also been attempted mainly from a synthesis of the sedimentological and palaeobotanical literature. Thus the Cambrian–Triassic surface albedo variations shown in Fig. 2 should be as accurate as is possible from the current literature. To ensure comparability with the work of Barron *et al.*⁸ a plot of $Q(1-a_T)$ is given (Fig. 2) rather than albedo, where Q is the total solar radiation received over the total area of a latitudinal belt and a_T is the surface albedo. Thus $Q(1-a_T)$ is the amount of absorbed radiation at the surface of the Earth.

Values of albedo for various surface types and sea at different latitudes are those given in Barron *et al.*⁸. Snow is not prescribed as there is little geological evidence for such cover. Prescription of snow cover between 60° and 90° for the early Carboniferous decreases the $Q(1-a_T)$ from 11.883 to 11.771. All land is prescribed a desert albedo before the Devonian. The first land plants are probably late Silurian¹⁶ and although lichens may have covered the early Palaeozoic land surface there is no evidence for this and a desert landscape is considered more probable. The advent of abundant land plants during the Devonian decreased the surface albedo (increased $Q(1-a_T)$) appreciably (Fig. 2).

Thus the early Palaeozoic was a time of high surface albedo with $Q(1-a_T)$ values similar to or lower than those of today (Fig. 3). However, major early Palaeozoic glacial periods are known only in the late Ordovician–medial Silurian¹⁷ of Africa and South America. Probable early Ordovician glaciation in Argentina is localized¹⁸ and there are no records of a medial Ordovician glaciation. Geographically restricted ice caps were probably present in South America and, perhaps locally, in Africa^{17–19,20}. A surprisingly high $Q(1-a_T)$ value is evident during the Devonian reducing to slightly lower values during the Carboniferous. However there is no obvious correlation between high surface albedo (low $Q(1-a_T)$) values and the onset of the major glacial periods during the Ordovician, Carboniferous and Tertiary.

If the latitudinal distribution of the land and sea is plotted (Fig. 3) for epochs preceeding ice-age initiation then no obvious correlation is found.

A low latitude terrestrial aggregation occurred in the late Cambrian (Fig. 3c) and certainly reduced $Q(1-a_T)$ (Fig. 2).

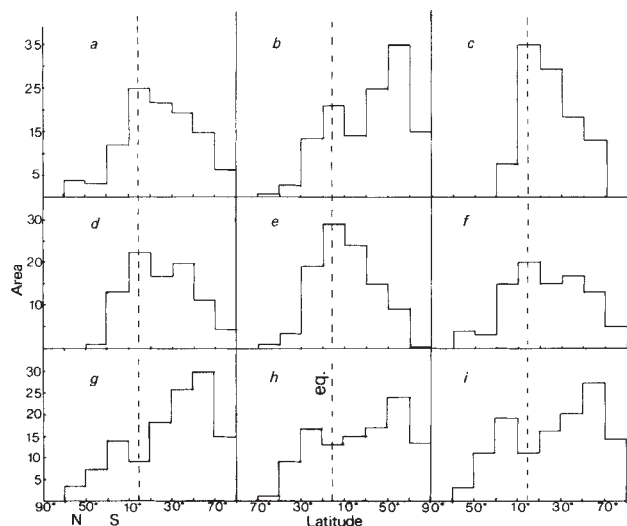


Fig. 3 Histograms of land area with respect to 20° palaeolatitude increments. Area $\times 10^6$ km². eq, Equator; a, early Cambrian; b, medial Cambrian; c, late Cambrian; d, early Ordovician; e, medial Ordovician; f, late Ordovician; g, early Carboniferous; h, medial Carboniferous; i, late Carboniferous.

However, no major ice cap was formed during the late Cambrian or early Ordovician although this may be due to a lack of high latitude land (Fig. 3c,d). Another near-equatorial aggregation of land occurred during the medial Ordovician (Fig. 3e), lowering the $Q(1-a_T)$ value and was succeeded by the major late Ordovician ice age at high latitudes. Thus the Cambro-Ordovician data suggest that ice-cap initiation requires both high albedos, generated by deserts at low latitudes, and sufficient land at high latitudes for ice to accumulate. An increase in absorbed radiation in the mid-Silurian (Fig. 2) may have been sufficient to end the ice-age. Unfortunately, such a scenario cannot be applied to the initiation of the late Carboniferous-Permian glaciation. Low surface albedo values preceded the glacial event during the Devonian and early Carboniferous mainly due to a relative dearth of low latitude land (Fig. 3). Similarly no obviously special configuration of land preceded the initiation of the southern ice-cap during the Oligocene^{8,21}. Thus the data presented in Figs 2-4 show that no obviously simple correlation exists between the latitudinal distribution of land and total surface albedo for epochs immediately preceding the initiation of ice-caps and a more complex explanation involving cloud-cover albedo and interactions with other major climate determinations may have to be developed.

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Climatic conditions deduced from a 150-kyr oxygen isotope-pollen record from the Arabian Sea

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The correlation reported here of oxygen-18 and pollen records from off-shore marine cores¹ enables the history of the nearby continental vegetation, which is controlled by climate, to be examined within the frame of the standard oxygen isotope stratigraphy. Core MD 76 135 from the northern Arabian Sea recovered sediments ranging from oxygen isotope stage 1, Holocene, to stage 6 before 128 kyr BP. Compared with the distribution of modern pollen, low sea-level glacial intervals contain well-preserved pollen which indicate saline littoral, arid and steppe inland conditions. High sea-level interglacial intervals are characterized by savanna-type vegetation. Pollens which are climatically diagnostic and wind-transported indicate distant source areas and reflect the intensification of low-level opposite air flows: the summer south-west monsoonal flow during the last interglacial and the beginning of the present interglacial, and the winter north-east trade winds during the last glacial maximum.

In present climatic conditions, the monsoon system dominates the atmospheric circulation over the Arabian Sea with seasonal low-level wind reversal following the latitudinal migration of the Intertropical Convergence Zone. In winter (October-May), steady but not very strong, dry north-east trade winds blow from Asia (Fig. 1). In summer (May-October), the cool, moist, steady and stronger southwesterlies, which are fed by the Southern Hemisphere winter south-east trade winds^{2,3}, bring heavy monsoonal rainfall to the south and east Asiatic continent^{4,5} (Fig. 1).

These low-level winds transport pollen from the continents to the ocean where it is eventually deposited in deep-sea sediments. Interpretation of pollen spectra in the Arabian Sea sediments relies on the present-day seasonal distribution of pollen in the air over the Arabian Sea, which identifies both its geographical source area and the wind responsible for its transport. We have studied this present-day distribution by collecting pollen with air filters on board ships travelling along the coasts^{6,7}. The cotton gauze filters are impregnated with silicon oil which collects and retains the mineral particles and pollen grains. They are renewed as often as necessary according to the nearby continental vegetation zones. In Table 1 we summarize data from the coast of Somalia during the summer monsoon and the winter trades. The pollen percentages refer to the total pollen.

Along the coast of Somalia, a 10-day record in July-August shows that the low-level south-west wind carries the highest percentages of Cyperaceae, Euphorbiaceae, *Podocarpus* and *Olea*. These taxa are derived from East Africa where the rainfall follows the migration of the Intertropical Convergence Zone of the tropical easterlies, and from the Ethiopian Highlands where rainfall originates in the African monsoon from the Atlantic Ocean⁸. They are well-dispersed East African pollen types^{9,10}.

Along the coast of Arabia, a 7-day record in November yielded mostly Chenopodiaceae and *Artemisia* pollen grains. The north and north-east low-level air flow suggests an origin in the Arabian desert and the Beluch and Iranian plateaux. The Thar desert does not appear as a source area, as in its southern

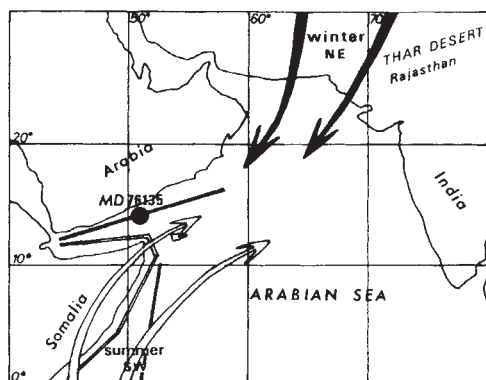


Fig. 1 Location of core MD76135. Arrows indicate low-level seasonal wind flow. Lines indicate ship-routes with pollen collection on air-filters. Solid line: winter air-filters (cruise Orgon IV of R/V Jean Charcot in November 1977, cruise Osiris IV of M/S Marion Dufresne in November 1979). Double lines: summer air-filters (cruise Osiris IV of M/S Marion Dufresne in July–August 1979).

part, the pollen rain does not include large amounts of these taxa¹¹.

As already observed in the Sahara¹², aeolian pollen transport is effective over very long distances, and its influence is dominant in arid zones. There, ground dust and pollen are resuspended in the atmosphere by diurnal thermal turbulence, uplifted to 2,000 m or more, and then transported by strong (but not necessarily prevailing) winds¹³. Over the Atlantic, dust of Sahara desert origin has been estimated¹⁴ at 260×10^6 tons per year. There is no estimate of the amount of dust from Somali, Arabian and Thar deserts over the Arabian Sea, although the importance of wind in the transport of clay minerals and quartz to the Arabian Sea bottom is recognized¹⁵. The high frequency of haze (solid aerosol) over the north-west Arabian Sea in summer¹⁶ indicates mobilization by thermal turbulence, and the low-level steady strong south-west flow¹⁷ suggests a Somali origin. However, occasional north-west storms from Arabia⁴ probably contribute to the haze. In winter, the haze disappears¹⁶. The steady, weak, low-level winds from the north, north-east and north-west would transport quartz dust from the Thar and Arabian deserts into the Arabian Sea¹⁸. The low-level air flow probably picks up a great deal of pollen from the littoral zone. Accumulated over several seasons in the inland deserts, dust and pollen would be uplifted to higher altitudes and then, feeding the low-level flow, settle progressively on the sea surface.

This preliminary present-day aeolian pollen survey, which is the means to interpret the climatic variations from the pollen record in Arabian Sea sediments, shows that the two conspicuously opposite climatic and wind-regime patterns are traceable by pollen: pollen collected in summer off Somalia indicates a south-west origin, and in winter off Arabia, a north, north-east and north-west origin.

To document the response of this monsoonal system to the changing boundary conditions during the late Quaternary, we studied core MD76135, collected at $14^\circ 26'6''$ N, $50^\circ 31'3''$ E, 1,895 m water depth (Fig. 1). We have measured the $^{18}\text{O}/^{16}\text{O}$ ratio of the planktonic foraminiferal species *Globigerinoides ruber* and the benthic foraminiferal species *Cibicides wuellerstorfi*. Analyses were performed using a Micromass 602C mass spectrometer and an experimental procedure described elsewhere¹⁹. Results are expressed as $\delta^{18}\text{O}$ defined by the relationship:

$$\delta^{18}\text{O} = \left[\frac{(^{18}\text{O}/^{16}\text{O})_{\text{sample}}}{(^{18}\text{O}/^{16}\text{O})_{\text{PDB}}} - 1 \right] \times 1,000$$

where PDB is the Chicago PDB-1 standard. No correction has been applied to take account of *Cibicides* departure from isotopic equilibrium²⁰ as this single benthic species has been analysed continuously. Twenty-nine samples were routinely

treated for pollen analysis²¹ and yielded an average of 250 pollens each.

The isotopic records of the planktonic and benthic foraminifera show, as usual, several oscillations. These $\delta^{18}\text{O}$ variations closely match those of other cores from the Atlantic²², Pacific and Indian Oceans²³, in agreement with the hypothesis that the major signal in these records reflects isotopic changes in the ocean water mass resulting from the waxing and waning of continental ice sheets²⁴.

Following Emiliani²², interglacial stages are identified by odd integers increasing with age and glacial stages are identified by even integers also increasing with age. Isotopic stage 2 corresponds to the last glacial maximum centred about 18 kyr ago, whereas stage 5 represents the last interglacial as observed in equatorial areas²². Stage 5 has been divided into five substages, of which the oldest, 5e, correlates with the Eemian interglacial of north-west Europe^{25–27}. Isotopic substage 5e is poorly represented in core MD76135: while glacial isotopic stage 6 is well represented, there is a hiatus corresponding to the transition from stage 6 to interglacial substage 5e, and the lower part of the substage 5e. The upper part of substage 5e is represented by 10 cm of sediment. The sedimentary record is continuous above that level (Fig. 2).

Core MD76135 has provided a well documented pollen diagram. The relative abundance of pollen taxa is shown in Fig. 2. Dominant taxa include Chenopodiaceae which are arid and halophilous and can have a double source area, either inland desert²⁸ or littoral sebkhas²⁹, the dominant source in our marine record. Gramineae and Cyperaceae reflect the environment of the Sudanese savanna. These three dominant taxa originate in the near-shore and inland vegetation. The pollen diagram also contains various less abundant taxa which are presented in Table 2 according to their phyto-geographical source area and their ecology: humid tropical, from open woodland to mangrove, tropical montane (*Olea*, *Podocarpus*), from East Africa, Ethiopia and Yemen, Sudano-Sahelian savanna in Somalia, Sahelian near-shore steppe in Arabia and Somalia, Mediterranean sub-arid steppe (mostly *Artemisia*) from the Middle East plateaux. All the taxa are still extant in the area.

Figure 2 (right-hand side) shows variations in the pollen groups most diagnostic of climate and wind-regime (Table 2, first five columns): East African total humid tropical taxa carried by south-west winds, dry tropical Sahelian taxa from near-shore steppe, Mediterranean steppe taxa from the north-east. The three dominant taxa of the diagram are excluded (Gramineae, Cyperaceae, Chenopodiaceae). Thus the diagnostic taxa are expressed as percentages of a partial sum which represents only ~20% of the total pollen sum.

The comparison of the pollen and isotopic records of core MD76135 shows that interglacial substage 5e is marked by high relative frequencies of the Sudanese savanna and humid tropical pollen taxa which include the long distance transported tropical African Montane *Podocarpus* and *Olea* signalling a strong south-west monsoonal air flow, while Chenopodiaceae, Sahelian steppe and Mediterranean steppe *Artemisia* pollen decrease. We interpret this period to be both warm and humid. The lowest part of post-glacial isotopic stage 1, early Holocene, around 8 kyr BP at 130 cm core depth, is also characterized by

Table 1 Pollen data collected from the coast of Somalia

Month	Arabia	Somalia	
	November	November	July–August
Latitudes	11°5'N–16°4'N	1°0'N–10°3'N	11°N–0°3'N
Total pollen	1,363	701	1,681
% Chenopodiaceae	59.2	18.6	13.2
% <i>Artemisia</i>	24.9	3.1	0
% Cyperaceae	0	0.4	3.3
% <i>Podocarpus</i>			
+ <i>Olea</i>	0	0.1	1.9
% Euphorbiaceae	0.1	1.7	3.9
% Gramineae	6.5	38.6	54.1
% Others	9.3	37.5	23.6

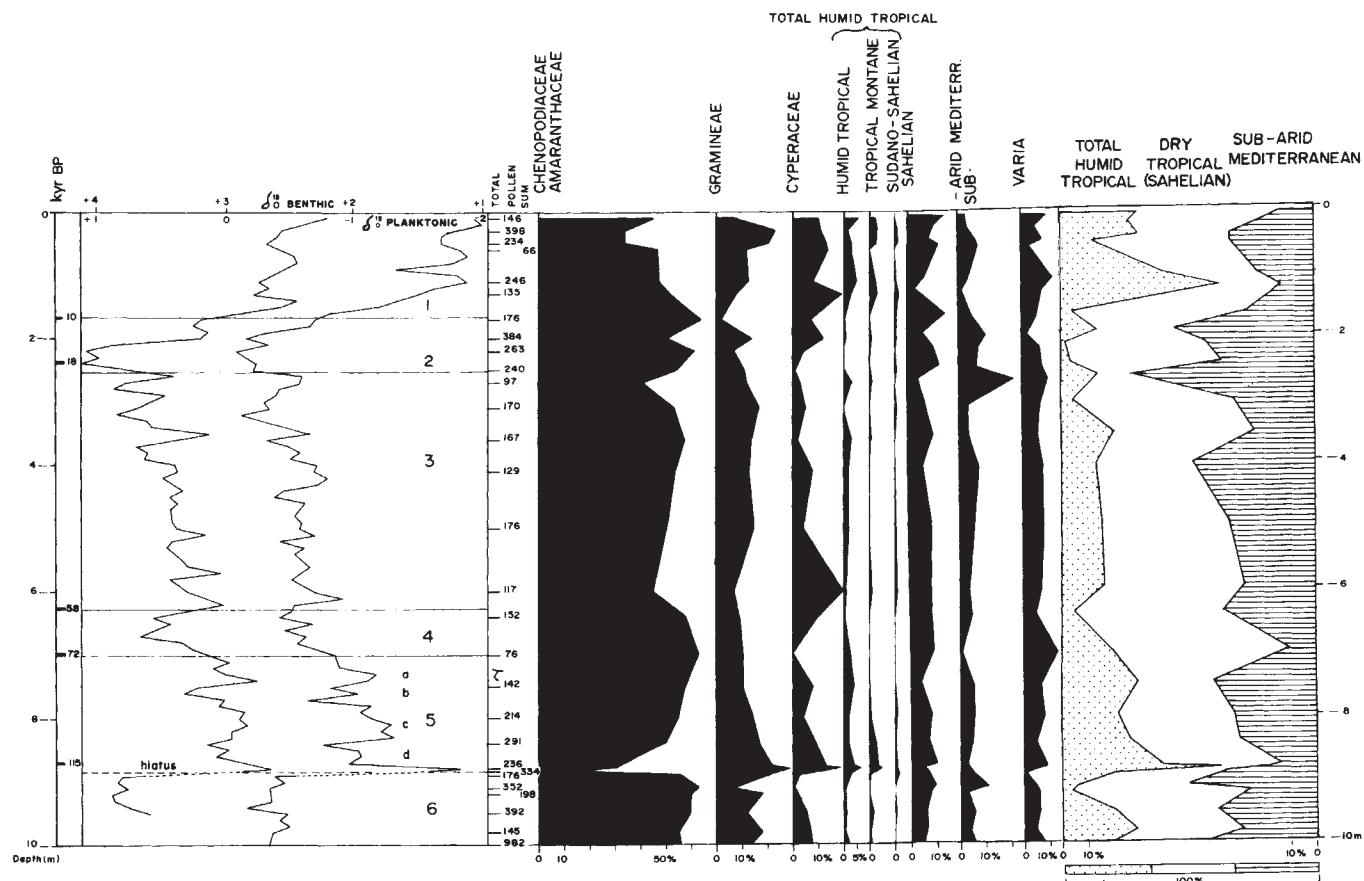


Fig. 2 Oxygen isotope and pollen record of core MD76135. Ages are estimated from refs 24, 41, 46. On the right column, total humid tropical taxa include humid tropical, tropical montane and Sudano-Sahelian taxa of the main diagram. All pollen relative frequencies are calculated on the total pollen sum.

highly increased amounts of humid tropical and Sudano-Sahelian savanna taxa, indicating a stronger south-west monsoonal flow, an increased Indian monsoon, and more moisture in Somalia. The late glacial-early Holocene (Bølling-Allerød to Boreal) was the most humid period since the last glacial maximum in all the equatorial latitudes of the planet³⁰, a response to the insolation peak in summer centred at 11 kyr BP, controlled by the variations of the Earth's orbital parameters³¹. In East Africa, this humid phase, clearly displayed by high lake levels and pollen records^{30,32-40}, begins at 12.5 kyr BP, and its peak is reached from 10 to 8 kyr BP. In our record, around 12.5 kyr BP, at 200 cm core depth immediately after the most arid last glacial

maximum at 220 cm core depth, there is a sign of locally increased moisture fostering the Somalia savanna, and of intensified long distance transport of humid tropical pollen. The same level of moisture and wind intensity is found during isotopic stage 3, while during isotopic stage 5, it is always significantly higher. Between the incipient humidification (around 12.5 kyr BP) and the Holocene main humid peak, the cold/dry Younger Dryas (11-10 kyr BP), clearly marked in Fig. 2 by the step of the isotopic curve at the transition of stages 2 to 1 (ref. 41), at 165-m core depth, correlates with local desiccation (decline of Gramineae-Cyperaceae, rise of Chenopodiaceae and Sahelian), and weakening of the monsoonal wind (decline of

Table 2 Taxa listed according to their phyto-geographical source area and ecology

Humid tropical	East African total humid tropical		Dry tropical sahelian	Sub-arid Mediterranean steppe	Varia
	Tropical montane	Sudano-Sahelian			
<i>Alchornea</i>	<i>Olea</i>	Acanthaceae	<i>Abutilon</i>	<i>Artemisia</i>	Anacardiaceae
Apocynaceae	<i>Podocarpus</i>	Capparidaceae	<i>Acacia</i>	<i>Calligonum</i>	<i>Betula</i>
<i>Avicennia</i>		<i>Celtis</i>	<i>Blepharis</i>	<i>Centaurea</i>	Caryophyllaceae
Combretaceae		<i>Dobera glabra</i>	<i>Caesalpinia</i>	Compositae lig.	<i>Cleome</i>
Meliaceae		<i>Hypoestes</i>	<i>Cassia</i>	<i>Ephedra</i>	<i>Convolvulus</i>
<i>Rhizophora</i>			<i>Commiphora</i>	<i>Erodium</i>	<i>Cordia</i>
<i>Sida</i>			Compositae tub.	Ombelliferae	<i>Corylus</i>
Spores			Cruciferae	<i>Plantago</i>	<i>Euphorbia</i>
			<i>Heliotropium</i>		<i>Fagonia</i>
			<i>Hyphaene</i>		<i>Fagus</i>
			<i>Indigofera</i>		Labiatae
			<i>Maerua</i>		<i>Myrica</i>
			<i>Phyllanthus</i>		<i>Nitraria</i>
			<i>Salvadora persica</i>		Papilionaceae
			<i>Tribulus</i>		Plumbaginaceae
			<i>Ziziphus</i>		<i>Pinus</i>
					<i>Rhus</i>

humid tropical). This is also widely displayed in Africa³⁰. In the western Arabian Sea, early Holocene faunal planktonic assemblages point to lower sea-surface temperatures and stronger south-west monsoon air flow⁴². Today, the summer south-west air flow induces strong upwelling along the Somalia and Arabia coasts, which is responsible for the extensive seasonal cooling of the Arabian Sea⁴³. In Rajasthan, north-west India, lake levels and pollen records point to the most humid monsoonal period between 10.3 and 9.5 kyr BP (ref. 44) still as humid but with more winter rain of Mediterranean origin from 9.5 kyr BP, until 5.0 kyr BP (refs 44, 45).

Glacial isotopic stages 6, 4, end of 3 and most clearly stage 2, the last glacial maximum are marked by low relative frequencies of humid tropical taxa and high relative frequencies of taxa from the Mediterranean steppe. These latter taxa originate from the north and north-east, indicating stronger north-east trade winds and increased aridity. High sea-surface temperatures estimated from faunal analysis in the western Arabian Sea during the last glacial maximum reflect reduced oceanic upwelling, as the south-west air flow was weaker⁴².

The arid halophilous Chenopodiaceae are abundant throughout the core and reflect mostly littoral vegetation²⁹. They decrease during interglacial isotopic substage 5e (together with the Sahelian steppe) and the upper part of stage 1 (late Holocene), when the sea level was high. However, in the Holocene, the most humid period as indicated by the highest relative frequency of savanna and humid tropical taxa, occurred at 130 cm core depth, before the Chenopodiaceae minimum at 50 cm core depth. During substages 5e to 5a, Chenopodiaceae increase while humid tropical taxa decrease. Glacial stages 6, 4 and 2, and also the transition from stage 2 to 1, Younger Dryas⁴¹, are characterized by high Chenopodiaceae and Sahelian steppe and low humid tropical taxa values, pointing to conditions drier than those of today. It is suggested that Chenopodiaceae reflect the salinity increase of the littoral water-table during glacial periods²⁹. Aridity lowers the freshwater table level in the newly uncovered, lower, regressive littoral strip. The seawater, which is then more saline, impinges for hydrostatic readjustment. Extensive littoral sebkhas vegetated by Chenopodiaceae result. This accounts for the very good positive correlation of Chenopodiaceae with the $\delta^{18}\text{O}$ enrichment and explains why the Chenopodiaceae Holocene minimum (maximum transgression) occurs significantly later than the Holocene most humid period.

We thus conclude that this marine pollen diagram from the arid tropical zone depicts the fluctuations of the intensity of the seasonally reversing low latitude wind systems, and of the local moisture, and correlates littoral aridity with sea-level variations. These fluctuations correlate with the global climate changes as indicated by isotopic stratigraphy. The intensity of the monsoonal winds seems to respond mainly to the variations of the insolation, controlled by the Earth orbital parameters, being maximum when the insolation is strongest⁴⁶. Glacial periods were arid in southwestern Asia and at low latitudes, and the north-east trade winds were intensified against a decreased monsoonal flow. In the tropics, as evidenced by the most detailed Holocene record, incipient interglacial stages were the most humid periods: the south-west monsoonal flow was intensified, over the Atlantic and Africa, and over the Arabian Sea and India. The insolation peak developed low pressures over the tropical continents and heavy rains resulted. Meanwhile, the middle and high latitudes, more influenced by the northern ice-sheets, also had warm summers⁴⁷ although dry, and reached their humidity peak later.

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A-nor-steranes, a novel class of sedimentary hydrocarbons

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As part of our investigations of Cretaceous black shales¹ we have analysed samples from a location near Aqualagna in the north central Apennines, Italy (43°39' N, 12°43' E). The saturated hydrocarbon fraction of one sample, a dark-grey shale of Upper Barremian age (115 Myr BP), contains novel series of steranes, which have been identified as C₂₆–C₂₈ 5β(H)- and 5α(H)-A-nor-steranes. Because these compounds are structurally related to 3β-hydroxymethyl-A-nor-steranes which have been found in some sponges, we suggest here that these steranes might be biological markers for certain types of sponges.

The Upper Barremian sample has an organic carbon content of 9% and was Soxhlet extracted with toluene/methanol (1/3,

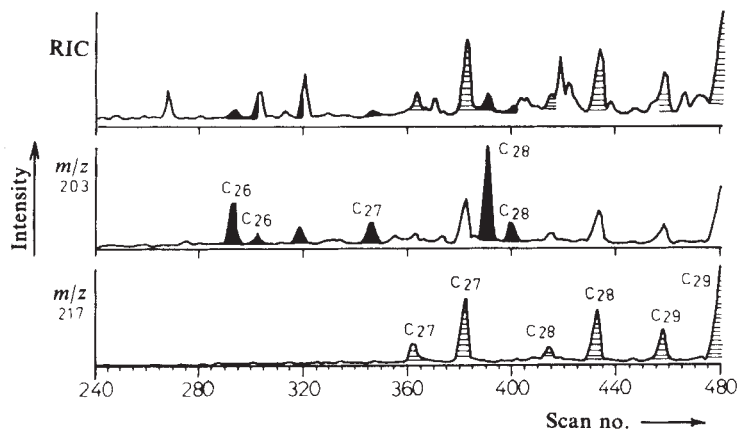


Fig. 1 Part of the reconstructed ion chromatogram (RIC) and of mass fragmentograms of m/z 203 (indicative of A-nor-steranes) and m/z 217 (indicative of steranes). Temperature programme: 150–280 °C (4 °C min⁻¹).

v/v; 20 h). The hexane-soluble part of the extract was separated by column chromatography over silica/alumina using hexane, toluene and toluene/methanol (1/1, v/v). The saturated hydrocarbons were isolated from the hexane fraction by Ag⁺-TLC (Merck silicagel impregnated with a 3% solution of silver nitrate in water/methanol) using pentane as a developer. Gas chromatography-mass spectrometry was performed on a Varian 3700 gas chromatograph equipped with a 25-m glass capillary column (i.d. 0.25 mm) coated with CP-sil-5 coupled to a MAT 44 quadrupole-mass spectrometer operating at 70 eV (mass range: m/z 50–500; cycle time, 2 s).

The saturated hydrocarbon fraction of the shale sample contains series of *n*- and isoprenoid alkanes, steranes, 4-methylsteranes and hopanes. Also present are a series of compounds with mass spectra that show a main fragment ion at m/z 203 (Fig. 1) and molecular ions at m/z 358 (Fig. 2a), 372 and 386. These mass spectra are similar to those of steranes with m/z 217, 218 shifted to m/z 203, 204 respectively and m/z 149 shifted to m/z 135.

Based on the mass spectra we assumed the unknown compounds to be a series of modified C₂₆, C₂₇ and C₂₈ steranes with 14 mass units missing from the nucleus and with side chains comparable to the common C₂₇, C₂₈ and C₂₉ steranes. From the shift of the fragment ion m/z 217—in steranes representing the ABC-ring part of the molecule²—to m/z 203 and the shift of the fragment ion m/z 149—in steranes ascribed to an AB-ring fragment³—to m/z 135 it can be deduced that a methylene group from ring A or B or the C₁₉ methyl group is not present in these compounds. The presence of a characteristic peak at m/z 262 in the spectrum of the C₂₆ unknown compound suggests that the missing carbon atom is the 19-methyl group or a methylene group of ring A. Ion m/z 262 in the spectrum of cholestane results from B-ring cleavage⁴ and therefore a substantial change

in the intensity of m/z 262 should be expected in the case of a B-nor-sterane.

To test our hypothesis the following compounds were synthesized^{5–9} (conversion of 19-hydroxycholest-4-en-3-one into 19-nor-cholesterol was achieved according to a reaction scheme obtained from Organon) (Fig. 3): 19-nor-cholesterol (the synthesis yielded three compounds with identical mass spectra, probably A–B ring junction isomers), 5 α (H)-A-nor-cholesterol and 5 β (H)-A-nor-cholesterol. The mass spectra of the 19-nor-cholesterols differed from those of the A-nor-cholesterols in the presence of a prominent peak at m/z 148 (Fig. 2d). The mass spectra of the 5 α (H)- and 5 β (H)-isomers of A-nor-cholesterol are different with regard to the ratios of M⁺ and M⁺ – 15 (Fig. 2b, c).

Mass spectrometry (Fig. 2) and coinjection on a capillary column coated with SE-52 showed that the first eluting C₂₆-unknown compound (Fig. 1) is identical to 5 β (H)-A-nor-cholesterol. The second unknown C₂₆-compound coeluted with 5 α (H)-A-nor-cholesterol but the mass spectrum of this less abundant unknown was not intense enough to compare it in detail with the spectrum of the synthetic compound. Therefore the second eluting C₂₆-compound is tentatively identified as 5 α (H)-A-nor-cholesterol. From these results it seems likely that the hydrocarbon fraction of the shale sample under investigation contains series of C₂₆, C₂₇ and C₂₈ 5 α (H)- and 5 β (H)-A-nor-steranes.

In addition to these 5 α (H)- and 5 β (H)-A-nor-steranes there is one other relatively intense peak in the fragmentogram of m/z 203 (Fig. 1, scan 318). The mass spectrum of this compound does indeed show an intense peak at m/z 203 but its identity is unknown at present. An assignment of this compound to a C₂₆-epimer of an A-nor-sterane seems very unlikely as the regular steranes in this sample are exclusively present in the natural 20R configuration.

It is now clear that the nor-steranes in DSDP sediments previously assigned tentatively as 19-nor-components¹⁰ are in fact A-nor-steranes (J. McEvoy, personal communication).

The unsaturated hydrocarbon fraction of the sample contains diasterenes, hopenes and in addition a series of compounds with mass spectra similar to those of diasterenes but with a base peak at m/z 243 instead of m/z 257 (ref. 11). Considering the presence of A-nor-steranes in the saturated hydrocarbon fraction we suggest that this series of unsaturated compounds is a series of A-nor-diasterenes. However, this suggestion has to be confirmed through the synthesis of the proper compounds.

As abiotic ring contraction of six-membered rings has not been reported in sediments, the presence of A-nor-steranes in the sample under investigation is thought to be the result of the input of related compounds showing this skeleton.

Sterols with a 5 α (H)-A-nor-nucleus and a 3 β -hydroxymethyl group have been found as major sterols in several sponges^{12–14}. With one exception these sponges all belong to the family Axinellidae¹⁴. It is not known, however, whether these sponges already existed during the Cretaceous. The A-nor-sterols are

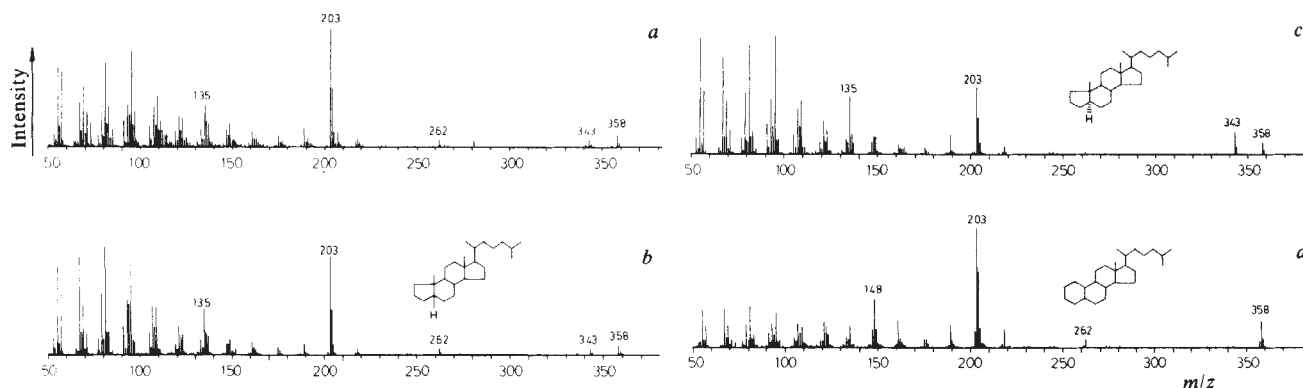


Fig. 2 Mass spectra of a, the first eluting unknown C₂₆-compound (scan 293, Fig. 1); b, synthetic 5 β (H)-A-nor-cholesterol; c, synthetic 5 α (H)-A-nor-cholesterol; d, synthetic 19-nor-cholesterol.

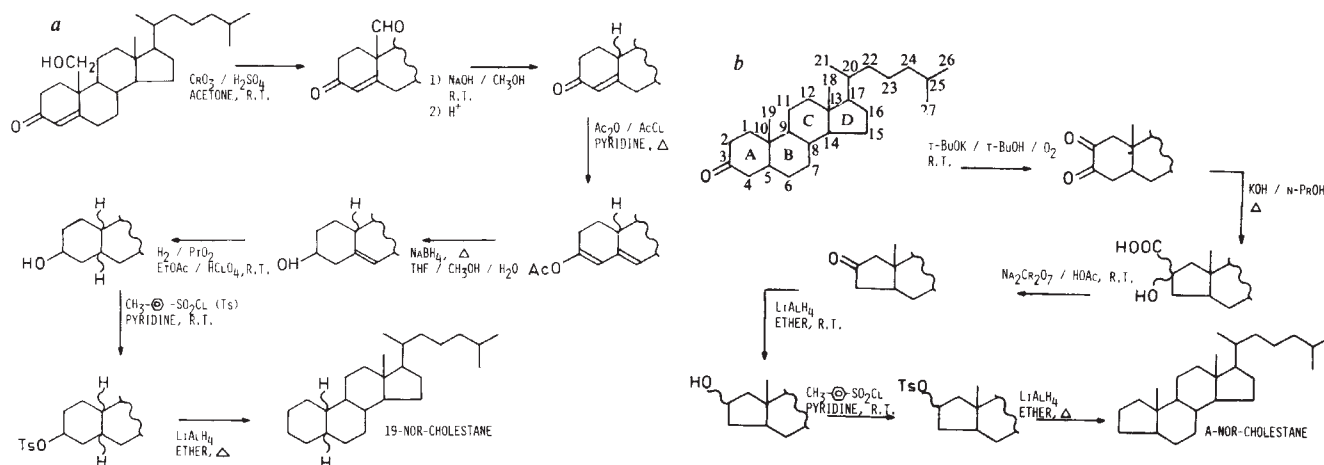
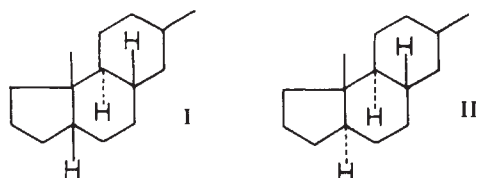


Fig. 3 Synthetic pathways for: *a*, 19-nor-cholestane from 19-hydroxy-cholest-4-en-3-one (refs 6–8); *b*, A-nor-cholestane ($5\alpha(\text{H})$ and $5\beta(\text{H})$) from cholestanone ($5\alpha(\text{H})$ and $5\beta(\text{H})$) (refs 6–9).

formed by the sponges through ring contraction of the dietary sterols¹⁵. It seems possible that the A-nor-steranes present in this Cretaceous sample are the ultimate diagenetic products of 3β -hydroxymethyl-A-nor-steranes, assuming that the 3β -hydroxymethyl group is removed (through microbial action?) and that isomerization at C_5 occurs at some stage of diagenesis resulting preferentially in the more stable $5\beta(\text{H})$ -isomer.



Molecular mechanics calculations using the EAS force field¹⁶ by J. M. A. Baas (Delft University of Technology) showed that model compound I (A-B *cis*) is thermodynamically more stable than model compound II (A-B *trans*) by 1.9 kcal mol⁻¹ (Fig. 4). See also calculations on the 8-methyl-hydrindanes in ref. 17. The presence of sponges seems to contradict the supposedly anoxic environment of sedimentation of black shales. However, some sponges are tolerant of very low levels of oxygen¹⁸. Moreover, black shale formation does not necessarily imply the existence of a thick anoxic water layer but can also occur when anoxic conditions are restricted to the sediment itself or to a thin (few millimetres) water layer at the bottom. We therefore suggest that these novel series of sedimentary hydrocarbons might be useful markers for certain types of sponges.

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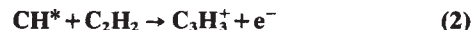
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Chemi-ionization in oxyacetylene flames

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The identity of the first ion formed in hydrocarbon flames has created much discussion. Two species have been favoured^{1–7}, produced in the chemi-ionization processes:



Reaction (2) is usually taken to involve electronically excited CH, in which case reaction (2), like reaction (1) with ground state CH, is close to thermoneutral. Experimental evidence has been claimed for both. For example, Peeters *et al.*³ prefer reaction (1), while Calcote *et al.*⁴ have suggested that reaction (2) is necessary to explain the abundance of ions in fuel-rich flames. Interestingly, evidence for CHO^+ has been obtained^{1,3,5} from either non-acetylene flames or those with low C/O ratios, while evidence for C_3H_3^+ appears^{2,4,6,7} in fuel-rich acetylene flames, where $[\text{C}_2\text{H}_2]$ is high and $[\text{O}]$ is low. We describe here a mass spectrometric study of fuel-rich acetylene flames, and attempt to clarify the picture. Evidence is presented which suggests that both reactions operate simultaneously in oxyacetylene flames.

Experimental details have been given elsewhere⁸. Briefly, pre-mixed laminar fuel-rich acetylene/oxygen/argon flames were burnt at atmospheric pressure on a single quartz tube. We are concerned here with three such flames, all having similar velocities and temperatures (~2,300 K), but with C/O ratios of 0.80, 1.02 and 1.09. With C/O = 1.02 and 1.09 a 'feather', that is, an extended luminous mantle, is visible downstream of the main reaction zone. Soot is only produced in the flame with C/O = 1.09. Ion concentrations were measured along each flame axis using a quadrupole mass spectrometer. Such concentration profiles could be obtained for single ions, or, by removing the d.c. component of the rod voltage, of the total ion current.

Various classes of positive ion emerge in this study. All hydrocarbon ions C_xH_y^+ reach peak concentration in the reaction zone or just downstream of it. In fact, they are of two types: aromatic ions, which decay extremely rapidly in the feather, and polyacetylene ions, which persist as far as the feather does and then decay quickly in the burnt gases.

However, major oxygen-containing ions, including H_3O^+ , CHO^+ and $\text{C}_2\text{H}_5\text{OH}_2^+$, have concentrations which peak in the burnt gases and then persist for several millimetres downstream. This is typified by the profiles for C_3H_3^+ and CHO^+ shown in Fig. 1. Figure 1a is for $\text{C}/\text{O} = 0.80$, where no feather is present. C_3H_3^+ , which is the most abundant of the C_xH_y^+ species, reaches peak concentration in the reaction zone and disappears very rapidly in the burnt gases, while CHO^+ peaks some 3 mm downstream of the reaction zone. As the fuel-oxygen mixture is enriched, the feather appears for $\text{C}/\text{O} > 0.9$; with $\text{C}/\text{O} = 1.02$ (Fig. 1b) the feather is ~ 5 mm long. C_3H_3^+ still peaks in the reaction zone (RZ) and is present throughout the feather, peaking again before decaying at the feather tip. However, CHO^+ reaches maximum concentration downstream of the feather tip, and is not detected at all in the reaction zone. Soot is actually seen in the richest flame studied here ($\text{C}/\text{O} = 1.09$, Fig. 1c), which makes the length of the feather difficult to estimate. However, CHO^+ peaks some 10 mm downstream of the reaction zone. Thus can an ion which is in such low concentrations and only seen in detectable quantities so far downstream, really be the only primary chemi-ion?

Figure 2, a plot of total positive ion current against distance, provides more information. All profiles are undoubtedly double-peaked, noticeable more as a shoulder for $\text{C}/\text{O} = 0.8$. The first peak is in the reaction zone, while the second appears just downstream of the feather tip. In fact, these two peaks correspond spatially to maxima for the ions C_3H_3^+ and CHO^+ .

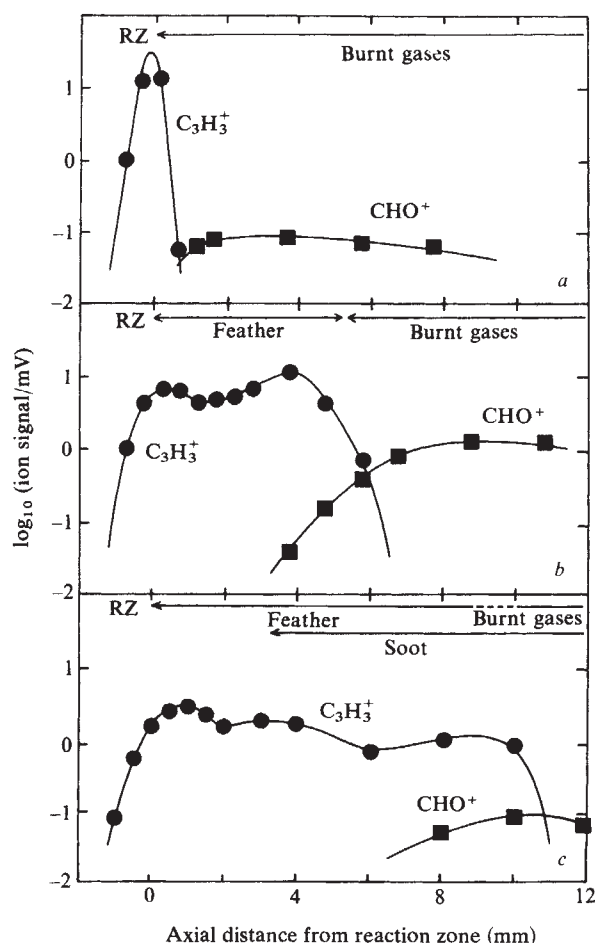


Fig. 1 Concentration profiles of C_3H_3^+ (●) and CHO^+ (■) in acetylene/oxygen/argon flames plotted as $\log_{10}$ (ion signal) against distance along flame axis. Zero distance corresponds to the primary reaction zone (RZ), as denoted by the emission of C_2 . Flames are: a, unburnt gas composition (by volume): $[\text{C}_2\text{H}_2]/[\text{O}_2]/[\text{Ar}] = 0.80/1.0/6.4$, so that $\text{C}/\text{O} = 0.80$; b, $[\text{C}_2\text{H}_2]/[\text{O}_2]/[\text{Ar}] = 1.02/1.0/5.3$, $\text{C}/\text{O} = 1.02$; c, $[\text{C}_2\text{H}_2]/[\text{O}_2]/[\text{Ar}] = 1.09/1.0/5.8$, $\text{C}/\text{O} = 1.09$.

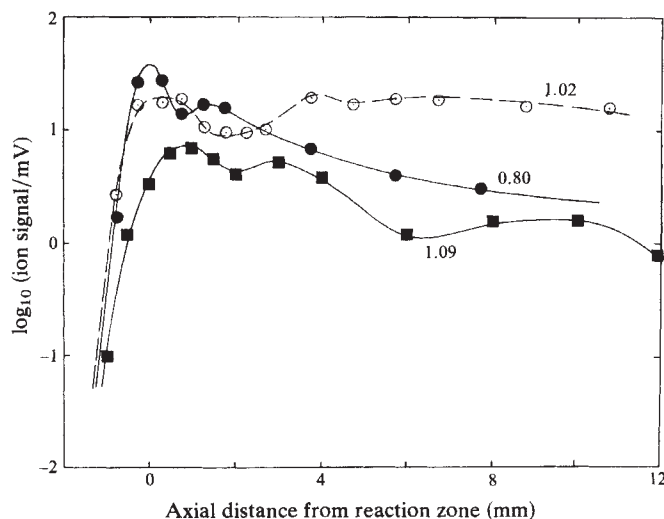
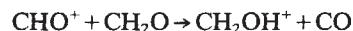


Fig. 2 Total positive ion intensity profiles in acetylene/oxygen/argon flames, with C/O ratios as shown, plotted as $\log_{10}$ (ion signal) against axial distance.

The second increase in total ion current indicates a fresh production of ions by a different mechanism, rather than chemical production from existing hydrocarbon ions. The profile plotted in Fig. 2 for the sooting flame ($\text{C}/\text{O} = 1.09$) is actually the sum of masses 0–170 AMU, rather than the total abundance of all positive ions. There is a distinction between these two quantities in this flame, which arises from the presence (but in sooty flames only) of positive ions having masses beyond the range of this instrument. The form of Fig. 2 avoids any complications from these heavy ions.

The two maxima in Fig. 2 and their relationship with the profiles of C_3H_3^+ and CHO^+ in Fig. 1 lead us to propose that two primary ionization mechanisms operate in fuel-rich acetylene flames, namely reactions (1) and (2). As the C/O ratio is increased, it is expected that the concentration of acetylene will grow, while oxygen atoms will decrease. Consequently, the rate of reaction (1) ($\propto [\text{CH}][\text{O}]$) will decrease, while that of reaction (2) ($\propto [\text{CH}^*][\text{C}_2\text{H}_2]$) will increase. The evidence above suggests that reaction (2) can become faster than reaction (1), although both are operating. The result is two separated zones of ion production. In lean acetylene flames and systems using other fuels, $[\text{C}_2\text{H}_2]$ will probably be low enough for reaction (1) to dominate. In fact, in very lean acetylene flames ($\text{C}/\text{O} = 0.06$), very little C_3H_3^+ is observed at all⁹. With CHO^+ as primary ion, hydrocarbon ions may be produced by processes such as:



This may explain the second (more downstream) peak in the profile of the hydrocarbon ions.

These results confirm and expand the work of Tse *et al.*⁶, who reported that (1) CHO^+ and H_3O^+ peak concentrations were a long way downstream of C_3H_3^+ , and (2) hydrocarbon ions were pyrolytic in origin and preceded true oxidation. The chemistry of neutral radicals in the feather seems to have received little attention in the past, although much is known of stable products^{10,11}. Ogden¹² has made emission spectroscopic measurements directly on CH^* and indirectly on H, OH and O; they indicate that $[\text{CH}^*]$ has a double-peaked profile which maximizes in the reaction zone and again early in the feather, while H, OH and O maximize in concentration just once, further downstream in the feather. However, as excitation of CH is chemical rather than thermal, no inferences should be made concerning ground state CH, which participates in reaction (1). Some quantitative data on this species would be useful in testing the viability of two chemi-ionization processes operating simultaneously. Even so, our conclusion that there

are two chemi-ionization processes agrees with what is known about the concentration profiles of the relevant neutral species.

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Preservation of unsaturated fatty acids in Palaeogene angiosperm fruits and seeds

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Despite the rapidity with which unsaturated fatty acids (UFAs) undergo autooxidation by atmospheric oxygen, Priestley *et al.*¹ have reported that oleic and linoleic acids persist in *Zea mays* seeds >1,500 yr old. They suggest, on the basis of analyses of progressively older seed samples (78–1,700 yr old), that a small proportion of UFAs may persist in seed tissues over extended periods of time. Here we report chemical analyses of Eocene and Oligocene angiosperm fruits and seeds (30–50 × Myr) which indicate the preservation of significant amounts of unsaturated fatty acids (9.6–17.8% of the total lipid content) in these ancient organic remains. The fossils contain larger proportions of polar unsaturated fatty acids, derived primarily from membranes, than those derived from storage lipids, suggesting a preferential oxidation of unsaturated fatty acid constituents of the membranes. We suggest that morphological characters, such as thin seed coats and the accessibility of the inner seed structure to the environment, may influence the extent of unsaturated fatty acid preservation.

Fossil material was collected from two localities of different age (Table 1). Seeds of *Zanthoxylum ornatum* were obtained

from the Middle Eocene (~45 Myr old) Geiselthal Flora of East Germany². Other fossils were collected from the Brandon Lignite of West-Central Vermont, the flora of which is now being investigated^{3,4}. The age of the Brandon flora is uncertain, but is presently inferred to be Middle to Late Oligocene (30 Myr old). In both cases the fossils were found as compactions (three-dimensional structures composed of original organic material) preserved in brown coal. These coals are the result of the compression of peat deposits in the swampy environments in or around which the parent plants grew. The fossils most probably were preserved in a waterlogged, anaerobic environment since the time of their deposition. The preservation is excellent in all cases, for example, seeds of *Zanthoxylum rhabdospermum* and *Euodia costata* retain the non-lignified secondary thickenings of the inner wall of the inner integument⁵. Four of the five fossils examined are seeds. Of these, *Z. ornatum*², *Z. rhabdospermum* and *Euodia lignita*⁵ possess sclerotic, semi-vitreous seed coats of 8–30 cell layers, 150–700 µm thick (Fig. 1a). In contrast, the seed coat of *Vitis macrochalaza*⁴ is thinner (1–2 cell layers, 60–225 µm thick), and is more leathery than vitreous^{6,7}. *Symplocos* sp., in contrast to the other fossils examined, is a trilobular endocarp bearing remnants of thin-walled seeds. Each locule opens to the exterior through a large pore, exposing the inner as well as the outer face of the fossil to ground water.

Representative fossils were collected from moist lignite, stored in glass vials, and subsequently crushed to a fine powder; to prevent oxidation of potentially preserved UFAs, we added an antioxidant (2,6-di-*tert*-butyl-*p*-cresol; 50 µg ml⁻¹). Extraction of fatty acids and hydrocarbons used the saponification procedure of Farrington and Quin⁸. Samples of the fossil material were refluxed (1 h) with 0.5 M KOH in 95% methanol/benzene (1:1 v/v). Non-saponifiable lipids were separated from saponifiable compounds by adding water to the saponification solvent mixture and extracting with petroleum ether. The aqueous phase of this separation was acidified and saponified lipids were extracted with petroleum ether. Fatty acids were isolated by methylating the dried residue of the saponifiable lipid extract using the BF₃-MeOH procedure of Metcalfe *et al.*⁹. Polar lipids (phospho- and glycolipids) were separated from storage lipids using the analytical technique of Priestley *et al.*¹⁰ and Dittmer and Willis¹¹. Fatty acid methyl esters were extracted with petroleum ether and purified by TLC on silica gel G plates. Identification of UFAs used combined gas and mass spectroscopy.

Microbial activity in the fossil specimens examined was evidenced by the detection of trace amounts of isomeric *cis*-vaccenic acid (18:1w7), which is an abundant bacterial constituent¹². Mass spectroscopy, however, revealed that the most prevalent 18:2 acid in our samples was 18:1w9 oleic acid. From comparisons of microbially decomposed seed tissues of some of the fossil taxa found as fossils, and the geolipids isolated

Fig. 1 a, Scanning electron micrograph of the seed wall of a broken *Z. rhabdospermum* seed (Brandon, Vermont). The spirally-thickened cells of the inner integument (at bottom) are succeeded by those of the bilayered outer integument, the inner portion of which consists of approximately five levels of brick-like cells and the outer of four to five levels of isodiametric sclereids. Scale bar, 50 µm. b, Scanning electron micrograph of the base of a trilobular endocarp of *Symplocos* sp. (Brandon, Vermont). Note the three pores, each of which leads to a locule carrying the remains of a thin-walled seed. Scale bar, 1 µm.

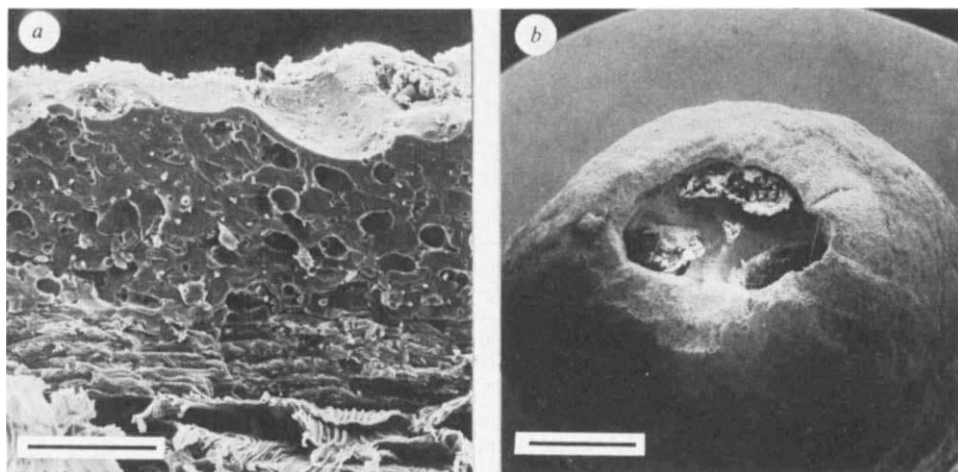


Table 1 Palaeobotanical data for fossil specimens examined

Specimen	Family	Locality	Age	Average wall thickness (μm)	No. of cell layers
<i>Zanthoxylum ornatum</i> Chandler	Rutaceae	Geiselthal, East Germany	Middle Eocene	300–700	20–30+
<i>Zanthoxylum rhabdospermum</i> Tiffney	Rutaceae	Brandon Lignite, Vermont	Middle Oligocene?	200–400	8–20
<i>Euodia costata</i> Tiffney	Rutaceae	Brandon Lignite, Vermont	Middle Oligocene?	150–250	8–15
<i>Symplocos</i> sp.	Symplocaceae	Brandon Lignite, Vermont	Middle Oligocene?	650–1,000	14–20
<i>Vitis macrochalaza</i> Tiffney	Vitaceae	Brandon Lignite, Vermont	Middle Oligocene?	60–225	1–2

Table 2 Representative UFA content of fruits/seeds of the Brandon Lignite

Specimen	(mg per g seed)	% Total fatty acids				% Total unsaturated fatty acids†	
		16:0	18:0	18:1	18:2	Polar subfraction	Storage subfraction
<i>Zanthoxylum ornatum</i>	7.4* (0.5)	36.0* (0.1)	47.9 (0.1)	10.3 (0.2)	3.7 (0.5)	6.3	7.7
<i>Zanthoxylum rhabdospermum</i>	13.4 (0.3)	48.0 (0.5)	34.0 (0.2)	15.7 (0.3)	2.1 (0.3)	5.7	14.1
<i>Euodia costata</i>	109 (0.9)	35.2 (0)	43.2 (0)	9.6 (0)	4.8 (0)	5.5	8.9
<i>Symplocos</i> sp.	5.6 (0.7)	39.7 (0)	49.7 (0)	9.6 (0)	—	1.3	8.3
<i>Vitis macrochalaza</i>	7.7 (0.5)	40.3 (0)	44.8 (0)	8.4 (0)	4.0 (0)	5.6	6.8

Fatty acid methyl esters were identified on (1) a $2\text{ m} \times 1.5\text{ mm}$ glass column packed with 20% FFAP on 70–80 mesh crom W-AW-DMCS operated isothermally at 190°C , and (2) a $2\text{ m} \times 1.5\text{ mm}$ glass column packed with 15% diethylene glycol succinate on Anakrom ABS 110/120 mesh, operated isothermally at 145°C .

* Mean; range is shown in parentheses.

† Distribution of % total 18:1 and 18:2 acids in polar and storage lipid subfractions.

from the fossil specimens examined here and from their associated inorganic strata, we conclude that biotic contamination of our specimens was minimal. The geolipid (UFA) profiles presented are interpreted, therefore, as being representative of the endogenous biochemical profiles of the fossil taxa.

With the exception of *Symplocos*, the fossils contain significant percentages of linoleic ($\text{C}_{18:2}$) and oleic ($\text{C}_{18:1}$) acids (Table 2), indicating that complete oxidation of the UFA fraction has not occurred despite the age of the specimens. The lack of $\text{C}_{18:2}$ in the endocarp of *Symplocos* may be ascribed to the thin-walled nature of the seeds and their continuity with the external environment through the locular pores (Fig. 1b). While the relative proportion of palmitic acid ($\text{C}_{16:0}$) is greater than that of $\text{C}_{18:0}$ in *Zanthoxylum* seeds, the remaining specimens show a repeated pattern in the relative abundance of the four major acids detected ($\text{C}_{18:0} > \text{C}_{16:0} > \text{C}_{18:1} > \text{C}_{18:2}$). This differs from the relative proportion of these acids found in modern counterparts ($\text{C}_{18:2} > \text{C}_{18:1} > \text{C}_{18:0} > \text{C}_{16:0}$), and is interpreted as evidence for the enrichment of the $\text{C}_{18:0}$ acid component by the oxidation of $\text{C}_{18:2}$ and $\text{C}_{18:1}$ constituents. Consistent with this notion is the observation that *Symplocos* endocarps lack detectable $\text{C}_{18:2}$ and yield the highest percentage of $\text{C}_{18:0}$ (Table 2). A comparison of the proportion of fatty acids isolated from the polar lipids (predominantly of membrane origin in modern seeds) and from storage lipid subfractions (Table 2) provides evidence for the preferential degradation of membrane UFAs.

Our study of the UFA content of these fossils indicates that a degradation of the membrane UFA subfraction parallels the absence of preserved membrane structure. Transmission electron microscopy of fossil specimens from the Brandon Lignite revealed little or no demonstrable protoplasmic details. The deterioration of membranes may well have resulted from lipid peroxidation and the consequent perturbation of the characteristic biomolecular (lipid-protein) membrane infrastructure. Electron microscopy has demonstrated that some membrane structure survives in ancient seeds and leaf tissues, indicating that sufficient membrane lipids may persist to maintain the bilayer configuration^{13,14}. Priestley *et al.* reported that the UFAs of the storage lipid and membrane lipid subfractions of maize seeds 1,700 yr old were equally susceptible to oxidation. The apparent preferential preservation of storage lipid UFAs in the fossil material examined here could reflect some differential distribution of naturally occurring antioxidants in the tissues. The ability of tocopherols, in low concentrations, to prevent lipid peroxidation is well documented¹⁵. Gaillard¹⁶ has reviewed

the roles of antioxidants in lipid metabolism. Note that tocopherols accumulate in plastids and that their synthesis and that of storage lipids in seeds may be separate, independent processes. It is noteworthy that plastids appear to be the most persistent organelles in fossil leaf tissues^{13,14}.

The preservation of such labile components in fossil reproductive structures >50 Myr old, confirms the previous speculation¹ that a residual, long-lasting UFA component will survive in old seed tissues. It is also possible that other labile constituents of chemotaxonomic significance may be preserved in reproductive organs such as seeds and fruits from the localities examined. Geochemical analyses of other fossil specimens have revealed a vast array of biochemical constituents commonly thought to be too labile to survive over extended geological time¹⁷. The data presented here can be placed in the context of reports documenting the preservation of flavonoids¹⁸, chlorophyll derivatives¹⁹ and various phenolics²⁰ in comparably old fossils and fossil strata. Further chemical and ultrastructural analyses of Brandon Lignite fossils and specimens from other localities may elucidate the biochemical processes involved in fossilization as well as provide information relevant to the taxonomy of problematical angiosperm genera.

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Vision has a role in *Limulus* mating behaviour

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In 1928 Hartline noted that the lateral eye of the horseshoe crab, *Limulus polyphemus*, was an admirable preparation for research in visual physiology. Since that time, extensive studies of *Limulus* have yielded fundamental understandings of visual processes and of sensory processes in general^{1,2}. However, except for demonstrations of primitive phototaxis³⁻⁵, studies have not yet revealed a possible role of vision in the animal's behaviour. We have further investigated the problem by studying mating behaviour, the only known behaviour exhibited by *Limulus* in its natural habitat. We report here that horseshoe crabs discriminate form and contrast during mating: males are attracted to painted cement castings of the female carapace and other forms, the degree of attraction depending on the form and contrast of the castings, and on the time of day. The discrimination of form may result from information transmitted by several sensory systems but the discrimination of contrast requires vision.

Along the eastern coast of North America, *Limuli* move in from deep water in the spring and build nests on protected beaches near the water's edge at high tide⁶. Numerous female-male pairs arrive, together with an excess of males. We observed the activity of males in the vicinity of cement castings placed in the water at a nesting beach.

The cement castings were made from an adult female carapace (27 cm wide), a hemisphere (29.5 cm diameter) and a cube (1,640 cm³). The exposed surface areas of the hemisphere and cube were each equivalent to that of the female carapace (1,365 cm²). Each casting was painted black, grey or white, yielding relative contrasts against sand underwater of -0.70, +0.1 and +0.75, respectively. The average relative contrast of the *Limulus* carapace against sand was -0.33 ± 0.03 ($n = 12$). All contrast values were determined by reflection densitometry, using a reflection density guide (Kodak 146 5947) and panchromatic black and white film (Kodak Plus-X). The three different castings, together with three shades of paint, yielded nine experimental objects. Two of each object, giving a total of 18 castings, were placed in random order in a row 4 m below the high-water line on the south side of Mashnee Dike, Cape Cod, Massachusetts. We observed the behaviour of males in the vicinity of each casting during eight high tides, four at night and four during the day.

Figure 1a shows a male *Limulus* mounted on a black cement casting of the female carapace. The male has mounted the dorsal part of the casting in the usual position for egg fertilization. Some males maintained this position for several hours. Figure 1b shows a single male soon after it approached and made contact with the prosomal region of a grey casting. Figure 1c shows a male mounted on a white casting; four other males hover nearby, apparently searching for an opportunity to mount. The behaviour in Fig. 1 is indistinguishable from that of normal mating. Similar behaviour was observed in the vicinity of every cement casting, but the number of males around a specific casting depended on its form and contrast.

Figure 2a shows that the number of males was greatest around the female models and lowest around the cubes. In each of eight observation periods, males selected female models in preference to hemispheres and hemispheres in preference to cubes. The probability of these results occurring by chance is 3^{-8} ; $p < 0.001$. The degree of selection was not significantly influenced by time of day. We conclude that male *Limuli* can

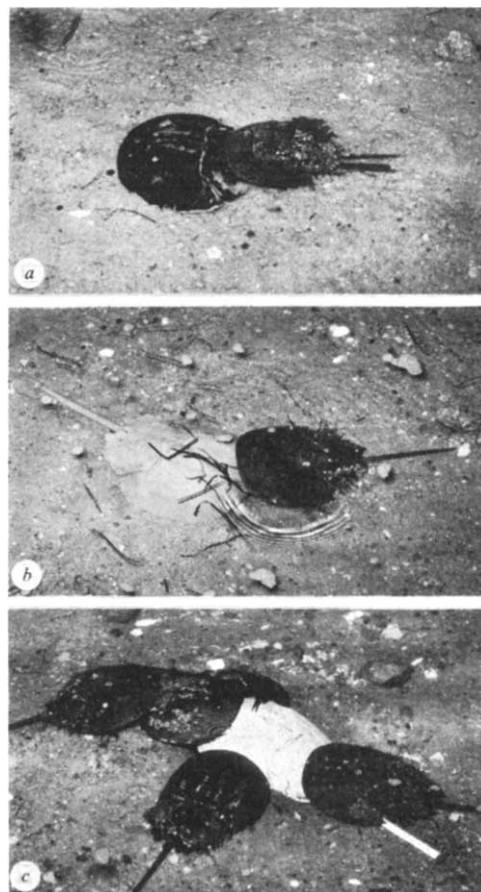


Fig. 1 Male horseshoe crabs in the vicinity of submerged, painted castings of the female carapace. *a*, A male *Limulus* mounted on the posterior portion of a black casting in the normal position for egg fertilization. *b*, A male in contact with a grey casting soon after approaching the model. *c*, One male mounted on a white casting with four others hovering nearby apparently searching for the appropriate mating position. All photographs were taken during the same night-time observation period with Kodak Plus-X film and flash. The animals and models are under water in each photograph. Note in *b* the ripples generated on the water surface by the male. The overall length of the black casting in *a* is 55 cm.

discriminate form. Discrimination of form may be based on tactile or visual inputs, or both.

Figure 2b shows that the contrast of a casting also influences male mating behaviour. The data on male contacts with castings were pooled according to the shade of paint used (black, grey or white). Black castings yielded the highest percentage (40%) of male contacts, regardless of time of day. The attraction of males to the grey castings was also high and almost equal to that of black castings during the day but not at night. Black and grey castings may have attracted most males during the day because they are similar in shade to the female carapace and are clearly visible. Note that the relative contrast of the female carapace against sand is -0.33, which falls within the contrast values of the black and grey castings (-0.7 and +0.1). At night, the low relative contrast of the grey castings against sand may have made them less visible. Indeed, the grey *Limulus* casting in Fig. 1c is difficult to distinguish from its underwater background. We could not see the grey castings at night but had no difficulty in detecting the white and black castings by illumination from either a new or full moon. The results in Fig. 2b suggest that, when visibility is not a limiting factor, male *Limuli* are attracted to objects that appear similar in shade to the female carapace.

Taken together, the data in Fig. 2a and b show that males were attracted most to the black *Limulus* casting and least to the white cube: in each of the eight observation periods, black *Limulus* castings yielded the highest number of male contacts and white cubes the lowest number.

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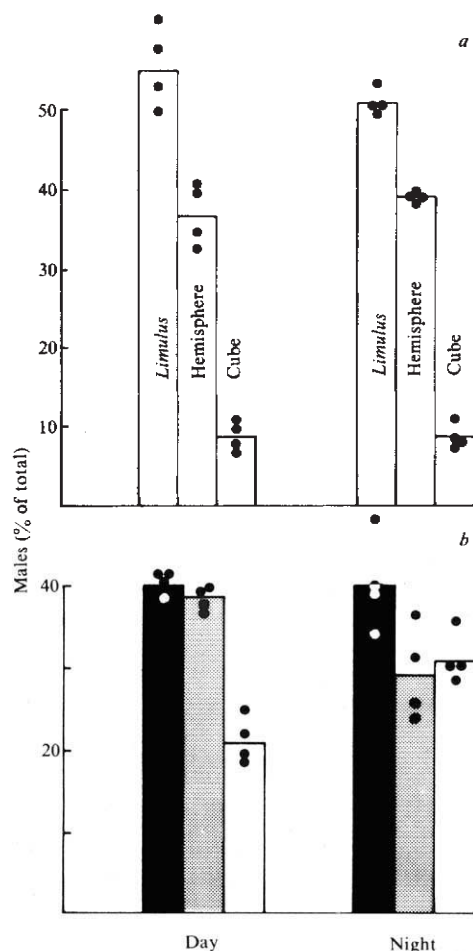


Fig. 2 Percentage of males observed at the cement castings during the day and at night. *a*, Filled circles indicate the percentage of males observed at each cement form during four daytime and four night-time observation periods. The daytime percentages did not differ significantly from the night-time values. Columns represent the average values. *b*, Filled and unfilled circles indicate the percentage of males in contact with the black (solid columns), grey (stippled columns) and white (open columns) castings during the same eight observation periods as in *a*. Columns represent the average values. The black castings attracted the greatest percentage of male *Limuli* regardless of time of day. The attraction of the grey castings was greater during the day, that of the white castings lower. Results are based on a total count of 6,988 males in contact with the set of 18 castings described in the text. The data were collected during eight high tides as males, in their search for females along the water's edge, approached and explored the submerged castings. Two observers separately surveyed the castings every 5 min during the 3-hr period that the tide covered the set of castings. The day- and night-time data were then summed and normalized.

Blinded animals were not attracted to the cement castings. In one night-time experiment we covered the median and lateral eyes of 75 male *Limuli* with black acrylic paint. The density of males around the castings fell by a factor of 8, and the distribution of the few animals found near the castings did not match that shown in Fig. 2. This result cannot be considered conclusive because some of the blinded animals buried themselves in the sand after being released at the water's edge. Nevertheless, the results are consistent with the notion that vision has a role in mating behaviour.

Visually guided behaviour is also evident from films taken during the day, which show that male *Limuli* passing within 2 m of a casting of a female carapace often change orientation and travel directly towards the casting, then explore and attempt to mount it.

Vision thus appears to play a part in *Limulus* mating behaviour, but other sense modalities may also be involved. Our

observations suggest that male animals are attracted to objects by form and contrast. Maintained contact with objects may be based on tactile cues transmitted by a well developed mechanoreceptor system^{7,8}. Chemical cues may also be involved⁹, but as yet no information on chemical attractants is available.

Circadian rhythms in the *Limulus* visual system¹⁰ may be an integral part of the mechanisms underlying the behaviour reported here. At night a circadian clock in the *Limulus* brain transmits efferent neural activity to the lateral eyes, increasing the response and decreasing the noise of single photoreceptor cells¹¹. Circadian changes in retinal physiology and anatomy increase the sensitivity of a dark-adapted lateral eye by as much as 30–100 times at night¹². High visual sensitivity may adapt the animal for specific nocturnal behaviour such as mating.

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Serotonin-activated adenylate cyclase during early development of *Schistosoma mansoni*

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Schistosoma mansoni infection is a major parasitic disease which afflicts 200 million people, mostly in developing countries. The parasite has a complex life cycle during which it is parasitic on a snail at the larval stage and on a mammalian host as an adult. In addition, there are two free-swimming stages: miracidia, which must penetrate the snail host, and cercariae, which leave the snail to find the correct mammalian host. On penetration into the mammalian host, the cercaria loses its tail and is referred to as a schistosomule. Adult stages of *Fasciola hepatica* (liver fluke)^{1,2} and *S. mansoni*³ have probably the highest adenylate cyclase activity reported, and in both parasites the enzyme can be activated by serotonin. Studies on *Fasciola*⁴ have shown that GTP is necessary for serotonin activation. One possible main function of high adenylate cyclase activity is the regulation by cyclic AMP of anaerobic glycolysis, which is the main source of energy in the adult stage of these parasites^{5,6}. We have compared the activities of adenylate cyclase in different developmental stages of *Schistosoma*. We report here that the cercariae have low adenylate cyclase activity which responds only poorly to activation by serotonin and GTP. However, in schistosomules cultures *in vitro*⁷, we found that within the first 4 days the cyclase system changed from one showing low serotonin activation, similar to that of cercariae, to one of high activity similar to that found in the adults.

Adenylate cyclase activity in adults was compared with that in cercariae. Particle preparations isolated at 2,000g contained

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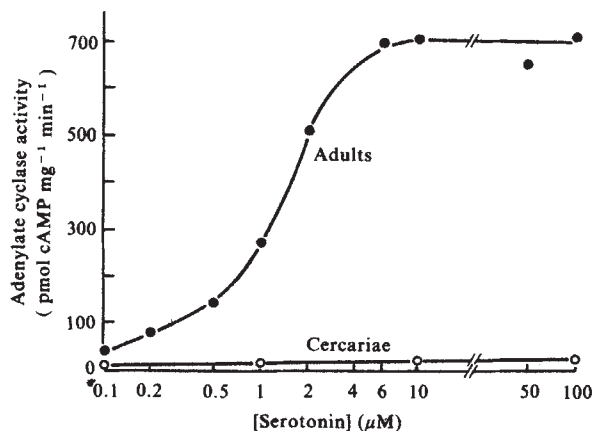


Fig. 1 Serotonin activation of adenylate cyclase from adult and cercaria particles. Representative experiment on adult (●) and cercaria particles (○) in which serotonin was added at the indicated concentrations to adenylate cyclase reaction mixture containing 0.1 mM ATP and 1×10^{-5} M GTP. Adenylate cyclase activity was determined as described in Table 1. Basal activity (without activators) for adults was 8 pmol cyclic AMP (cAMP) $\text{mg}^{-1} \text{min}^{-1}$ and <3 pmol cyclic AMP $\text{mg}^{-1} \text{min}^{-1}$ for cercariae.

almost all the cyclase activity. Representative experiments summarized in Table 1 show that when the enzyme was maximally activated with NaF, the specific activity of the cercarial enzyme was ~20% of that of the adult. Serotonin plus GTP caused almost no activation of the cercarial enzyme, whereas it markedly stimulated adult adenylate cyclase. Figure 1 shows the effect of various concentrations of serotonin plus GTP on the enzyme from cercariae and adults. The specific activity of the two enzymes is markedly different even at concentrations as low as 1–10 μM of serotonin. The results in Table 1 also show that the poorly hydrolysed GTP analogue, Gpp(NH)p (guanylyl imidophosphate), by itself stimulated the enzyme from both sources, whereas the natural nucleotide GTP had no stimulatory effect. Note that serotonin in the presence of Gpp(NH)p caused significant stimulation of enzyme activity

Table 1 Cyclic AMP (pmol $\text{mg}^{-1} \text{min}^{-1}$) produced in cercariae and adult *S. mansoni*

Activators	Cercariae	Adults
None	2	8
NaF (10 mM)	1,232	5,985
GTP (0.01 mM)	4	7
Serotonin (100 μM)		
+ GTP ($\times 10^{-5}$ M)	15	557
Gpp(NH)p (2×10^{-5} M)	66	440
Gpp(NH)p (2×10^{-5} M)		
+ Serotonin (100 μM)	213	1,798

Adult worms, obtained from hamsters by perfusion¹⁶, were homogenized using a glass homogenizer in 0.25 M sucrose, 5 mM dithiothreitol (DTT) and 1 mM EDTA. Snails were placed under light to induce shedding of cercariae. Cercariae were concentrated by cooling until swimming stopped, and then centrifuged. The pellet was homogenized in a glass homogenizer with a motorized glass-Teflon pestle in the same buffer as the adults. Both homogenates were spun twice at 2,000g for 15 min each. The final resuspended particles were used as the enzyme source. We determined adenylate cyclase activity using the method of Salomon *et al.*¹⁷, which measures the production of radioactively labelled cyclic AMP from [α -³²P]ATP. [α -³²P]ATP was added to the mixture after the reaction was stopped to monitor cyclic AMP recovery as described by Salomon *et al.*¹⁷ under 'method C'. Assays contained 0.1 M sucrose, 50 mM glycylglycine (pH 7.5), 0.5 mM isobutyl methylxanthine, 5 mM phosphocreatine, 2 mM MgCl_2 , 5 mM DTT, 0.02 mM EDTA, 0.1 mM Na_2ATP , 5 U creatinephosphokinase, and ~1 μCi [α -³²P]ATP. Reactions were initiated with 0.05 ml of particles and the final volume of the reaction mixture, with or without specific activators, was 0.25 ml. All samples were incubated for 10 min at 30 °C. Protein was determined by the method of Bradford¹⁸.

from the cercariae, though not as high as that of the adult. In these conditions of enzyme activation, the specific activity of the cercarial enzyme never exceeded 15% of that of the adult.

As there was such an obvious difference in total adenylate cyclase activity (with NaF) and serotonin-stimulated activity between adults and cercariae, the question arose of how rapidly the enzyme develops from low to high responsiveness to serotonin. We therefore studied the enzyme activity in the early intermediate stages of development from cercariae to adults. Schistosomules were prepared and cultured at 36 °C in a 5% CO_2 atmosphere for 10 days according to the procedure of Basch⁷, in medium containing 10% serum and 1 μM serotonin. Representative results for cyclase activity at different stages of development of the schistosomules are shown in Fig. 2. NaF-stimulated adenylate cyclase activity increased after preparation of the schistosomules from the cercariae; such an increase does not appear to be due to the experimental procedure used as preparation of the schistosomules by mechanical means⁸ without exposure to tissue culture media gave the same increase in fluoride-activated enzyme. The most significant change in the adenylate cyclase activity during schistosomule development was the marked increase in serotonin-activated enzyme on the second and fourth days (Fig. 2). While serotonin-activated adenylate cyclase in the cercariae was only 0.8% of NaF-stimulated activity, by the fourth day the adenylate cyclase in the schistosomules could be activated by serotonin to 6% of NaF-stimulated activity. Adenylate cyclase activity of schistosomules that were maintained in culture for up to 10 days showed no further increase in sensitivity to serotonin activation. Enzyme from adult worms grown *in vitro* also showed activation by serotonin to 6% of total activity (NaF-stimulated), although total activity doubled (Fig. 2). Serotonin-activated cyclase activity was higher in the parasites grown and collected from the hamster host than in schistosomules or in adults grown entirely *in vitro* (Fig. 2).

We then tested the effect of serotonin activation in the presence of Gpp(NH)p. Figure 3 shows that serotonin in the presence of Gpp(NH)p caused significant activation of cyclase in cercariae and schistosomules, an effect not shared by the natural nucleotide, GTP. Further activation by serotonin had doubled by the second day of schistosomule culture, when it reached a plateau. Note that much of the serotonin activity in

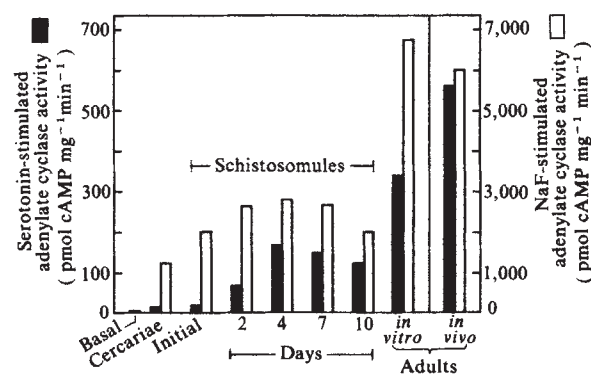


Fig. 2 Activation of adenylate cyclase by serotonin and GTP (left ordinate) and by NaF (right ordinate) at different stages of development. Cercariae from infected snails and adults from hamsters were collected as described in Table 1. Early schistosomules (initial) were isolated from cercariae and cultured according to the procedure of Basch⁷. Adults were prepared *in vitro* after culturing the schistosomules for 3 months, according to the Basch⁷ procedure. Preparation of particles as well as assay of adenylate cyclase were as described in Table 1 legend. Adenylate cyclase activity was measured in a reaction mixture containing 0.1 mM ATP and either 10 mM NaF or 100 μM serotonin with 10^{-5} M GTP. Adenylate cyclase activity in particles from schistosomules immediately after preparation from the cercariae is indicated by 'initial'. Basal activity for adults was <8 pmol cyclic AMP $\text{mg}^{-1} \text{min}^{-1}$ and for cercariae and schistosomules <3 pmol cyclic AMP $\text{mg}^{-1} \text{min}^{-1}$.

the presence of Gpp(NH)p in these stages of early development is due to the effect of the nucleotide itself. The adenylate cyclase activity of the adult worm (grown either *in vitro* or *in vivo*) was still higher than that in the early stages of development, indicating further increase in enzyme activity during development of the parasite to maturity. Serotonin has been reported to stimulate the motility of several trematodes including schistosomes (for review see ref. 9). In the present experiments we observed that cercariae, immediately after shedding their tails, did not respond to the stimulatory action of serotonin. However, after only 2 h in tissue culture medium, schistosomules responded fully to the stimulatory action of the indolamine on motility.

The cercariae of this trematode are short-lived free-living larvae whose main function is to find a suitable warm-blooded host. The results reported here clearly show that at this stage adenylate cyclase activity and its responsiveness to serotonin are extremely low. While the cercaria is a free-living organism in water, it develops to a schistosomule in the host, where it encounters serotonin. We have shown that early in schistosomule development, adenylate cyclase activity becomes higher and its responsiveness to serotonin markedly increases. The adult parasite showed an even greater increase in both total- and serotonin-stimulated adenylate cyclase activity. Thus as the parasite develops to maturity, it gains more adenylate cyclase that is sensitive to serotonin activation. Because of the marked difference between fully serotonin-activated cyclase activity and the maximum capability of the enzyme system in both schistosomules and adults, it is possible that other naturally occurring factors besides serotonin control cyclase activity during development of the parasite. The results suggest that adenylate cyclase is necessary to supply cyclic AMP early in development so as to achieve full parasite maturation. Cyclic AMP has been reported to be important for the development of trypanosomes¹⁰ and malarial parasites¹¹, as well as for cell differentiation¹² and proliferation¹³.

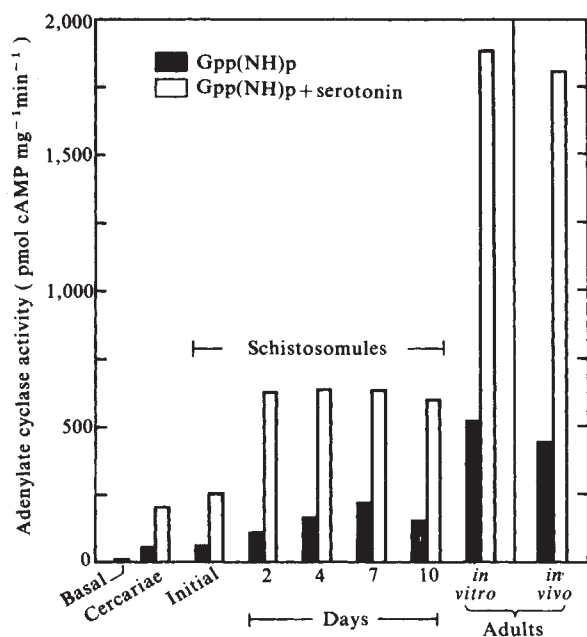


Fig. 3 Activation of adenylate cyclase from schistosomules by Gpp(NH)p with and without serotonin. Representative experiment on cercariae, schistosomules and *in vitro* and *in vivo* adults. Schistosomules and adult worms (*in vitro*) were obtained from cultures⁷, cercariae were obtained from infected snails as described in Table 1, and adults (*in vivo*) were dissected from infected hamsters. Particles were prepared and analysed as described in Table 1 legend. All adenylate cyclase reaction mixtures contained 0.1 mM ATP and 2×10^{-5} M Gpp(NH)p with or without 100 μ M serotonin, as indicated in Table 1. Basal activity for adults was <8 pmol cyclic AMP $\text{mg}^{-1} \text{min}^{-1}$ and for cercariae and schistosomules <3 pmol cyclic AMP $\text{mg}^{-1} \text{min}^{-1}$.

We reported previously that serotonin activates three key enzymes in the regulation of metabolism in a closely related parasite, *F. hepatica*: glycogen phosphorylase¹, phosphofructokinase⁶ and cyclic AMP-dependent protein kinase¹⁴. In all these enzyme systems, cyclic AMP appears to be involved in enzyme activation and therefore may be crucial for normal development of the parasite in the host. Apparently, the schistosomule synthesizes an adenylate cyclase that is more responsive to serotonin than the enzyme in cercariae. This developmental stage occurs in the first 48–96 h of the schistosomule life cycle, which corresponds closely to the time of complete skin penetration¹⁵ and arrival in the vascular system where serotonin is present. Serotonin-activated adenylate cyclase in the early stages of schistosomule development offers sites which are amenable to pharmacological manipulation and which could be used for the selection of more effective anti-schistosomal agents.

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Expression in *E. coli* of *Chlamydia trachomatis* antigen recognized during human infection

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Chlamydia trachomatis, a prokaryotic obligate intracellular parasite of eukaryotic cells, has long been recognized as the agent of trachoma, the major infectious cause of human blindness¹. In the past decade, clinical investigations have shown that the frequency and severity of chlamydial genital infection parallels that of gonorrhoea, and that *C. trachomatis* is a major cause of non-gonococcal urethritis, epididymitis, proctitis, cervicitis and salpingitis, as well as conjunctivitis and pneumonia in neonates². Despite the new awareness of its role in human disease, there is a need for improved methods of identification and control of *C. trachomatis* infections. Progress in these areas awaits a more detailed understanding of the pathogenesis of chlamydial infection, including definition of key antigenic structures recognized in human disease. We have studied the immune response to chlamydial polypeptides during human infection and have found that most infected patients have antibody to polypeptides of molecular weight (M_r) 67,000 (67K), 60K, 40K, 19K and 16K, regardless of infecting immunotype³. We report here the cloning and expression in *Escherichia coli* of the 19K *C. trachomatis* polypeptide, an immunogen in human chlamydial infection.

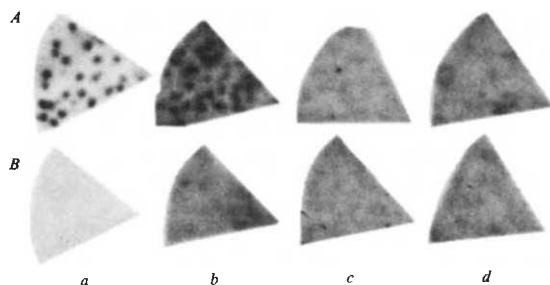


Fig. 1 Clone A4 plaques react specifically with serum from humans having chlamydial infection. Plates containing 100–150 clone A4 plaques were overlaid with nitrocellulose disks for 10–20 min. The disks were cut into quadrants then soaked in 5% OA-TSA (5% ovalbumin in 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.15% sodium azide) to saturate unused protein binding sites, and incubated overnight with individual sera diluted 1:250 in 1% OA-TSA. After washing, the disks were reacted with 125 I-protein A, washed, dried and exposed to Kodak X-Omat AR film between intensifying screens. **A**, reaction of A4 plaques with sera from infected (**a, b**) and uninfected individuals (**c, d**). **B**, reaction of λ 1059 plaques with the same four sera.

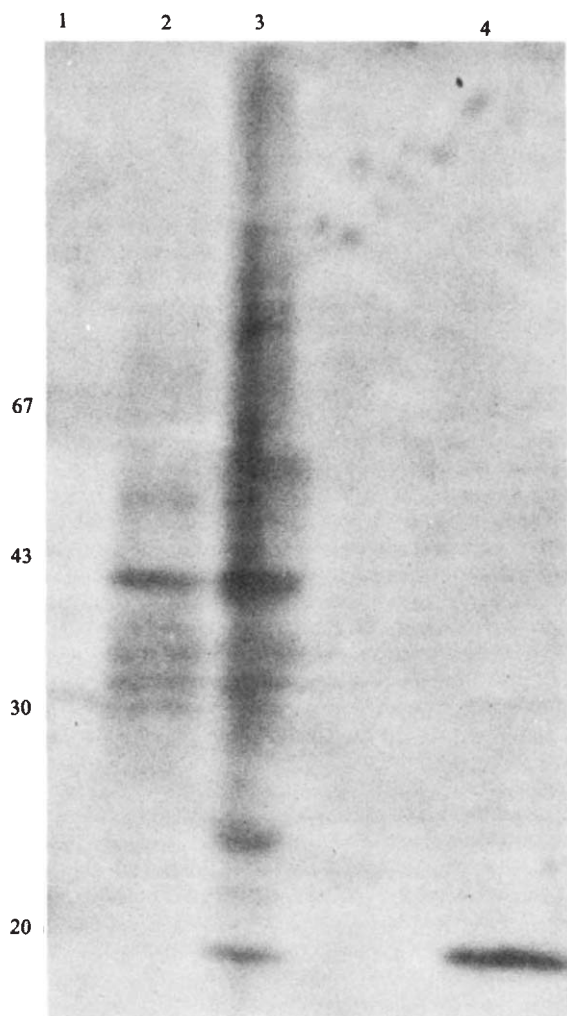


Fig. 2 Autoradiogram of clone A4 'Western' blot. Lysate samples were boiled in 0.0625 M Tris, 2% SDS, 10% glycerol, 0.001% bromophenol blue, 5% β -mercaptoethanol before loading on to 8–20% gradient polyacrylamide SDS gels. After electrophoresis and transfer to nitrocellulose, the blot was probed with the human chlamydial serum used in earlier screening (see Fig. 1a). Radioiodinated molecular weight standards (Pharmacia) are marked on the left ($\times 10^3$). Samples shown are host *E. coli* Q359 (lane 1), chlamydial L_1 elementary bodies (lanes 2, 3), and clone A4 grown in broth cultures of Q359 (lane 4). Except for lane 2, which was an older, partially degraded sample, approximately equal amounts of protein were applied to each lane.

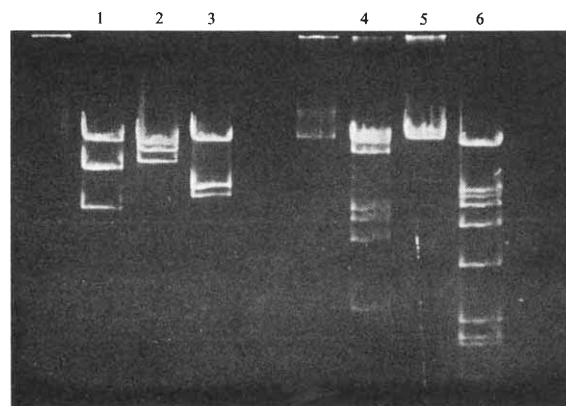


Fig. 3 Restriction endonuclease digests of vector and clone A4 DNA. DNA extracted from purified vector or recombinant phage was digested with various restriction endonucleases in recommended buffers (New England Biolabs). Cleaved DNA samples were then analysed on a 0.6% agarose gel and stained with ethidium bromide. Shown here are λ 1059 digested with *Eco*RI (lane 1), *Bam*HI-digested λ 1059 (lane 2), λ 1059/*Hind*III (lane 3), A4/*Eco*RI (lane 4), A4/*Bam*HI (lane 5), A4/*Hind*III (lane 6). Fragments were sized using λ /*Hind*III markers (BRL; data not shown). Unlabelled lanes contain nonreactive clone plaques.

Elementary bodies (EB) of *C. trachomatis* type L_1 , grown in HeLa cells, were purified through Renografin (Squibb) to yield EB free from host cell contamination⁴. Chlamydial DNA was extracted from EB in 20% sucrose containing 50 mM Tris pH 7.5, 20 mM Na_2EDTA , 200 $\mu\text{g ml}^{-1}$ proteinase K, and 0.7% Sarkosyl at 55 °C for 15 min, followed by further incubation at 37 °C for 45 min. After phenol extraction and equilibrium centrifugation in neutral CsCl , the chlamydial DNA was dialysed against 10 mM Tris pH 7.5, 0.1 mM Na_2EDTA . It was then partially digested with *Sau*3A I and ligated to *Bam*HI-cleaved vector phage λ 1059 (ref. 5). The ligated DNA was then packaged *in vitro*⁶ and used to transfect *E. coli* Q359 which is permissive only for recombinant plaques. Plaques were screened for expression of chlamydial antigens by an *in situ* radioimmunoassay.

Nitrocellulose disks were placed over the recombinant bacteriophage plaques to absorb plaque-derived protein. The disks were then exposed to serum from a human with chlamydial infection, followed by reaction with 125 I-labelled protein A of *Staphylococcus aureus* (Pharmacia) and autoradiography. One plaque which gave a particularly strong signal, designated A4, was chosen for further study (Fig. 1). Plaques of clone A4 reacted specifically with sera from patients having chlamydial infection but showed no reaction with sera from uninfected humans in conditions where plaques of vector λ 1059 (without a chlamydial DNA insert) did not react with sera from patients with chlamydial infection or from control patients.

The nature of the chlamydial antigen encoded by clone A4 was elucidated by 'Western blotting'⁷ of total protein from an A4 lysate (Fig. 2). A4 specifies a polypeptide antigen of M_r 19K (Fig. 2, lane 4), identical in size to that of an antigen of *C. trachomatis* elementary bodies.

DNA from clone A4 was partially characterized by restriction endonuclease cleavage (Fig. 3). As expected, clone A4 has a cleavage pattern which differs from that of the original vector λ 1059, consistent with an insert size of 15 kilobase (kb).

We have described here the expression in *E. coli* of a M_r 19 K antigen of *C. trachomatis*; we have shown previously³ that an antigen of this molecular weight is a consistent immunogen in human chlamydial infection. Antibody to this polypeptide is therefore indicative of chlamydial infection regardless of immunotype, and this could possibly become the basis of simple serotests for chlamydial infection. We believe that cloning the genes for major chlamydial immunogens in human infection provides a powerful tool for studying the immunobiology of this organism. It should be possible, using recombinant sources of

chlamydial antigens, to reproduce by experimental vaccination the pattern of antibody to chlamydial polypeptides found in various chlamydial diseases, and then determine whether this modifies experimental chlamydial disease.

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Transformation of the green alga *Chlamydomonas reinhardtii* with yeast DNA

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The development of a transformation system in the green unicellular alga *Chlamydomonas reinhardtii* is important for two reasons. First, a large number of nuclear mutants of this organism have been characterized, including auxotrophs, cytoplasmic and chloroplast ribosome mutants, photosynthetic and flagellar mutants^{1,2}. The isolation of wild-type genes from *C. reinhardtii* and from other plants may be possible by complementation with an efficient transformation system. Second, modification and reinsertion of these genes and their regulatory elements into *C. reinhardtii* cells is likely to provide important insights into their function and regulation. Here, we describe the first successful nuclear transformation of *C. reinhardtii*. A plasmid carrying the yeast *arg4* locus and a yeast replication origin^{3,4} has been used to transform a *C. reinhardtii* cell wall-deficient, arginine-requiring mutant. In several transformants the yeast gene appears to have integrated stably into the nuclear chromosomes.

The *arg7* locus of *C. reinhardtii* was used for selection. It codes for argininosuccinate lyase (ASL), the last enzyme of the arginine biosynthetic pathway, which converts argininosuccinate into arginine and fumarate⁵. In *C. reinhardtii* the enzyme consists of five (± 1) identical subunits of molecular weight ~39,000 (ref. 6). The first *arg7* mutant of *C. reinhardtii* was isolated by Gillham⁷; several other mutants of this type have since been obtained and characterized^{8,9}. The DNA used for transformation was the plasmid pYearg4 containing a yeast replication origin and the yeast *arg4* locus^{3,4}, which is analogous to the *C. reinhardtii arg7* locus. The 18 kilobase (kb) pYearg4 plasmid contains a single *EcoRI* site and eight *HindIII* sites and the *arg4* gene is located on the second largest (2.7 kb) *HindIII* fragment³ (fragment B in Fig. 2h). One important advantage of using this plasmid is that it cross-hybridizes only weakly with nuclear DNA of *C. reinhardtii*, thereby allowing an easy distinction between true transformants and revertants (see below). To avoid using cell wall-degrading enzymes to obtain protoplasts, the cell wall-deficient strain cw15 (ref. 10) was chosen. The double mutant cw15 *arg7* was constructed by Dr P. Bennoun.

Cells of cw15 *arg7* were treated with poly-L-ornithine¹¹ or polyethylene glycol (PEG), mixed with yeast plasmid and plated on plates containing acetate as sole carbon source¹² (see Fig. 1 legend for experimental details). After 2 weeks small green colonies appeared at a frequency of $\sim 10^{-6}$. Table 1 shows that the poly-L-ornithine method is more reproducible than the PEG method.

As the reversion frequency of *arg7* is $\sim 10^{-7}$ (ref. 9), the putative transformants were tested for the presence of yeast plasmid sequences. Total DNA was extracted from these cells and digested with restriction endonucleases. The DNA fragments were electrophoresed on agarose gels (Fig. 1), transferred to nitrocellulose filters according to Southern¹³ and hybridized with radioactively labelled pYearg4 plasmid DNA. Figure 1b shows that a 13.1 kb *EcoRI* fragment hybridizes with the probe in transformant T2-5. Hybridization of the same probe with undigested DNA produces a smear (Fig. 1d), indicating that the plasmid DNA is integrated in the nuclear DNA of this transformant. The possibility that the plasmid is not integrated but present as a high molecular weight concatemer can be excluded because pYearg4 hybridizes to a nuclear *HindIII* fragment that is larger than the largest *HindIII* fragment of pYearg4 (Fig. 2 lanes f, h). When total DNA from non-transformed cells is hybridized with the same probe, only one faint band, of lower molecular weight, is apparent (Fig. 1f). When nuclear DNA from non-transformed cells was mixed with one copy equivalent of pYearg4 plasmid per genome, cleaved with *EcoRI* (Fig. 1g) and hybridized to the probe (Fig. 1h) in order to estimate the copy number of integrated sequences, the intensity of the signal was comparable with that of Fig. 1b. Comparison of the sizes of pYearg4 (Fig. 1h) and the *EcoRI* fragment of the transformant, which hybridizes to the probe (Fig. 1b), reveals that at least 5 kb of the plasmid DNA have been deleted in the transformant. Similar hybridization patterns are obtained when the ColE1 vector alone or the purified *HindIII* fragment B of pYearg4 are used as probes (data not shown).

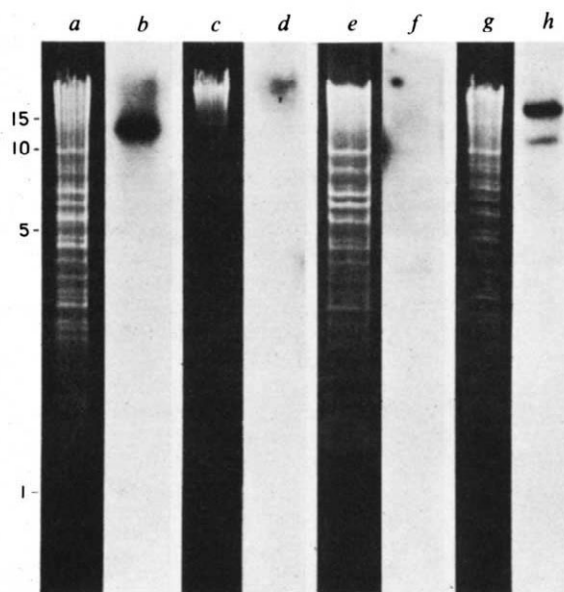
Figure 2a–c displays the hybridization patterns obtained with another transformant, T8-3. Whereas one major signal is visible with *EcoRI*- (Fig. 2a) or *HindIII*- (Fig. 2c) digested DNA, no strong band is detectable with undigested DNA (Fig. 2b), probably because of the poor transfer of larger fragments of DNA. As in the previous case it can be concluded from these data that a portion of the yeast plasmid has inserted itself into the nuclear DNA. Note that the *EcoRI* fragment of T8-3 hybridizing to the probe differs in size from the corresponding fragment of T2-5 and that the hybridization patterns obtained with *HindIII* digests of the DNAs of the two transformants are different (Fig. 2c, e, g). It appears therefore that the integration of pYearg4 in the two transformants has either taken place at different sites in the nuclear genome or at the same site with different deletions and/or that rearrangements of the plasmid sequences have occurred.

Table 1 Summary of transformation experiments

Expt	DNA used for transformation (5 µg)	No. of arg ⁺ cells	
		Method I	Method II
1	pYearg4	3	
2	pYearg4	2	3
3	pYearg4	2	0
4	pYearg4	1	0
5	pYearg4	4	0
7	pYearg4	4	0
8	pYearg4	3	2
9	pBR322-arg4	1	0

Methods I and II refer to the poly-L-ornithine and PEG transformation methods (see Fig. 1 legend). For each transformation 2×10^7 cells of cw15 *arg7*B were used. Cell survival was 50–80% in method I and 0–40% in method II. The pBR322-arg4 plasmid is described in Fig. 2 legend. In all cases 50 µg of salmon sperm DNA were included. In control experiments in which plasmid DNA was omitted, no Arg⁺ cells were recovered. Transformants T2-5 and T8-3 used in this study were obtained in experiments 2 (method II) and 8 (method I) respectively. Approximately one-half of the Arg⁺ cells examined by the Southern blotting technique displayed fragments hybridizing to the yeast DNA.

Fig. 1 Analysis of transformant T2-5. Two methods of transformation were used. I: 2×10^7 cells of *arg7* cw15B at a concentration of $<2 \times 10^6$ cells per ml were centrifuged at 3,000g for 5 min, washed once in Tris-acetate-phosphate (TAP), 0.2 M mannitol¹² and resuspended in 0.95 ml TAP, 0.4 M mannitol. Five to ten μ g of pYearg4 plasmid DNA³ were preincubated with 50 μ g carrier salmon sperm DNA and poly-L-ornithine in 5 mM ZnSO_4 ¹⁵ for 1 h on ice before being added to the cells. The final poly-L-ornithine concentration was 10 μ g ml⁻¹ and incubation was for 30 min at room temperature. Cells were washed three times in 5 ml TAP, 0.4 M mannitol; resuspended in 10 ml TAP, 0.2 M mannitol, 50 μ g ml⁻¹ arginine and placed on a shaker at 150 r.p.m. for 20 h under a light intensity of 3,000 lx. The cells were concentrated to 1 ml and 0.2 ml aliquots were mixed with 3 ml top agar (0.6% agar in TAP, 0.2 M mannitol)¹⁶ at 37 °C and poured on TAP plates (1.5% agar). Controls were treated identically except that the plasmid DNA was omitted. II: The second method is similar to the yeast transformation procedure¹⁷. After resuspension of the cells in 0.2 ml 10 mM CaCl_2 in TAP, 0.4 M mannitol, plasmid DNA (5–10 μ g) and carrier DNA (20–50 μ g) were added and the mixture was incubated at room temperature for 15 min. Two ml of 30% PEG-4000 in 10 mM Tris-HCl pH7.5, 10 mM CaCl_2 were added and the cells were incubated for 30 min at room temperature. The cells were washed and plated as in method I. The nuclear DNA was isolated from a 500 ml stationary phase culture. Cells were washed once with TAP growth medium¹² and resuspended in 0.1 M NaCl, 20 mM Tris-HCl pH8.0, 50 mM EDTA. SDS and pronase were added at a final concentration of 1% and 500 μ g ml⁻¹ respectively. Fresh pronase was added after 45 and 90 min. After 2.5 h incubation at 50 °C the lysate was cooled and extracted with distilled phenol. The aqueous phase was precipitated with 2 vol ethanol and centrifuged for 10 min at 10,000g. The pellet was washed once with 70% ethanol, dried and resuspended in 2 ml 10 mM Tris-HCl pH8.0, 1 mM EDTA overnight. The preparation was then treated with 50 μ g ml⁻¹ pancreatic RNase for 1 h at 37 °C. The DNA was centrifuged in an ethidium bromide density gradient (Beckman Ti50 rotor, 50 h at 35,000 r.p.m.) after inactivation of the enzyme with 0.05% diethyl pyrocarbonate for 5 min at room temperature. The DNA band was removed with a pipette, extracted twice with isoamylalcohol, precipitated with 3 vol 70% ethanol, rinsed in 70% ethanol and resuspended in 10 mM Tris-HCl pH 8.0, 1 mM EDTA. The agarose gel electrophoretic patterns are a, DNA of transformant T2-5 digested with *Eco*RI; c, T2-5 DNA without digestion; e, DNA from non-transformed cells digested with *Eco*RI; g, DNA from non-transformed cells mixed with pYearg4 DNA digested with *Eco*RI. The DNA fragments were blotted onto nitrocellulose filters¹³. b, d, f, h Represent autoradiographs of hybridizations of the DNAs shown in a, c, e, g respectively, using nick-translated¹⁸ pYearg4 plasmid DNA as a probe. The discrete bands in a, e and g represent chloroplast *Eco*RI fragments and the band in c, ~15 kb, is mitochondrial DNA^{19,20}. Numbers on the left indicate the sizes of the fragments in kb. Hybridizations were performed as described previously²¹ with minor modifications. Nitrocellulose filters carrying DNA fragments (16 $\times$ 16 cm) were prehybridized in 6 $\times$ SSC (1 $\times$ SSC = 0.15 M NaCl, 0.015 M Na citrate pH 7.0), 1 mM EDTA, 0.1% SDS, 0.2% Ficoll, 0.2% polyvinylpyrrolidone, 0.2% bovine serum albumin, 50 μ g ml⁻¹ salmon sperm DNA at 68 °C for 2–4 h. Hybridizations were in the same medium at 68 °C for 16–20 h except that the salmon sperm DNA concentration was raised to 1 mg ml⁻¹; 3×10^6 c.p.m. of ³²P-labelled hybridization probe were added. Filters were washed twice in 2 $\times$ SSC, 0.1% SDS at room temperature; four times for 1 h in 0.1 $\times$ SSC, 0.1% SDS at 52 °C; and 10 min in 3 mM Tris-base at room temperature. Filters were dried and exposed to Kodak Xs-5 films with tungsten intensifying screens at -70 °C.



Comparison of the sizes of the *Hind*III fragments of pYearg4 (Fig. 2h) with the hybridization patterns obtained with the *Hind*III digests of the DNAs from the transformants T8-3 (Fig. 2c) and T2-5 (Fig. 2f, g) confirms that a deletion exists in the integrated plasmid DNA relative to the free plasmid. As no hybridization occurs at the position of fragment B which contains the *arg4* locus, we conclude that a portion of this fragment has been fused to a nuclear DNA fragment. The fate of the smaller *Hind*III fragments of pYearg4 is more difficult to follow in the integrated state because the hybridization signals obtained with the probe are at the limit of detection.

Table 2 shows that the two transformants examined grow much more slowly than the non-arginine-requiring strain cw15. It also shows that the specific activity of ASL in crude extracts of the cw15 strain was 0.14 μ mol arginine per mg protein per h, considerably lower than the value reported for wild-type cells⁸. The activity of this enzyme is known to increase when the cells are grown in medium containing low concentrations of ammonium⁹; however, our cw15 strain and the transformants grow very poorly in these conditions and it was therefore necessary to use high concentrations of ammonium (400 mg l⁻¹) which repress the activity of the enzyme⁸. In addition, the enzyme may be subjected to more proteolysis due to the fragility of the cw 15 mutant cells. Table 2 shows that the ASL activities in the transformants are close to 5% of the value obtained in the cw15 strain, and are at the limit of detection. The yeast enzyme appears therefore to have a low activity in *C. reinhardtii* and/or be unstable in these cells. It is known, however, that *C. reinhardtii* can sustain a normal growth rate even if the level of ASL is low. While examining the interallelic complementation of several mutants of the *arg7* locus of *C. reinhardtii*, Loppes and Matagne⁹ found one diploid with a normal growth rate although the specific activity of ASL was only 5% of wild type. Besides interallelic complementation, it is also known that hybrid ASL can be formed between the products of wild-type and certain *arg7* mutant genes¹⁴. The question arises whether the ASL enzyme of the transformants consists uniquely of yeast subunits

or whether it is made functional by the interaction of the yeast and mutated *C. reinhardtii* subunits. Analysis of the heat sensitivity of the enzyme may distinguish between these two possibilities¹⁴, but we have not yet been able to perform such an experiment because of the low specific activity of the ASL enzyme in the transformants.

The stability of the transformants was examined by growing them for several generations in the presence of arginine. After 70 generations without selective pressure cells of T2-5 were still arginine independent. However, analysis of the integrated DNA by Southern blotting revealed changes in the hybridization pattern (Fig. 2). After 70 generations the *Eco*RI (compare Fig. 2d, e) and *Hind*III (compare Fig. 2f, g) fragments hybridizing to the probe have become smaller, suggesting that a segment of the integrated DNA has been deleted or that there has been a slight sequence rearrangement.

Because the transformation yield is still low it has been difficult to optimize the transformation procedure. It is not yet clear whether the limiting step is at the level of entry of DNA into the cells or at the level of stable integration of the DNA into the nuclear genome. Although the pYearg4 plasmid has been shown to contain a yeast replication origin and to generate high-frequency transformants in yeast⁴, we have no solid evidence that this replication origin is active in *C. reinhardtii*. Note,

Table 2 Properties of transformants

	Specific activity of ASL (μ mol arginine per mg protein per h)	Generation time (h)
cw15 <i>arg7</i>	—	14
cw15	0.14	7
T2-5	0.01	17
T8-3	0.01	23

The specific activities of ASL in crude extracts were determined as described in ref. 8. Cells were grown in Tris-acetate-phosphate (TAP) medium except cw15 *arg7* cells which were grown in TAP medium containing 50 μ g ml⁻¹ arginine.

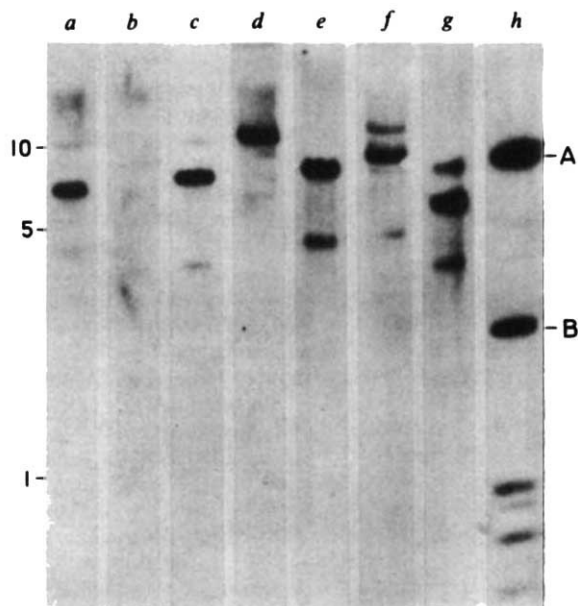


Fig. 2 Analysis of transformants. Preparation of the DNA of the transformants and the hybridizations of the DNA fragments with ^{32}P -labelled probes were performed as described in Fig. 1 legend. To obtain more specific hybridization to the *arg4* region, the *Hind*III fragment containing *arg4* (fragment B in lane h) was subcloned into pBR322. The new plasmid pBR322-*arg4* was nick-translated and used as probe in the hybridizations shown in a–c, e, g. The hybridization probe used in d, f and h was pYearg4. Lanes a, b, c represent autoradiographs of hybridizations of the probe to DNA of the transformant T8-3: a, *Eco*RI digest; b, undigested DNA; c, *Hind*III digest. Lanes d, f display hybridization patterns obtained with the DNA of T2-5 digested with *Eco*RI and *Hind*III respectively. Lanes e, g represent similar hybridizations with *Eco*RI and *Hind*III digests except that the DNA was extracted from T2-5 cells about 70 generations later than in d and f. Lane h displays the *Hind*III fragments of pYearg4. Fragments A and B contain the *ColE1* vector and the yeast *arg4* locus respectively³. Numbers on the left indicate the sizes of the fragments in kb.

however, that two putative slow growing transformants were shown initially, by the Southern blotting technique (data not shown), to harbour non-integrated DNA molecules hybridizing with the pYearg4 plasmid. After further growth of these clones subsequent analysis failed to show any DNA fragment hybridizing to the probe. It is possible that the strains initially carried an autonomously replicating plasmid, which was lost after reversion to arginine independence.

Despite these uncertainties the experiments described here clearly show that transformation is feasible in *C. reinhardtii* and that some of these transformants can be stably propagated. It will be important to develop suitable transformation vectors for *C. reinhardtii* in order to allow transformation to become an efficient method for isolating nuclear genes by complementation of appropriate mutants and for studying the regulation of expression of nuclear genes of *C. reinhardtii* and other plants.

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In vitro transformation of plant protoplasts with Ti-plasmid DNA

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Genetic engineering requires a procedure for introducing DNA into host cells, followed by integration into the host genome and gene expression. Although several procedures for DNA-mediated gene transfer in mammalian cells, yeast and bacteria have been reported, no such methods are yet available for plant cells. The major obstacle to DNA uptake in plant cells is the cell wall, but this can be circumvented by using plant protoplasts, cells freed of their cell walls by enzymatic digestion. However, none of the reports on the uptake of DNA into plant protoplasts^{1,2} has produced conclusive evidence for the integration of DNA into the host genome, that is, that stable transformation occurs. The tumour-inducing bacterium *Agrobacterium tumefaciens*, which causes crown gall disease, is a natural system for the introduction of foreign DNA into plants. This bacterium introduces part of its tumour-inducing (Ti) plasmid, called T-DNA, into plant cells, where it becomes integrated into the nuclear DNA of the host^{3,4} and is transcribed into mRNA^{5,6}. T-DNA encodes tumour-specific enzymes responsible for the formation of amino acid derivatives such as octopine or nopaline⁷, which the bacterium can use as a sole source of carbon and nitrogen. The transformed cells have also acquired the ability to grow in the absence of phytohormones (autotrophy). An *in vitro* system for infection of *Nicotiana tabacum* protoplasts by *A. tumefaciens* has already been reported⁸. Transformants are selected by their ability to divide and grow in tissue culture without the addition of plant phytohormones to the synthetic culture medium. Here, we report a reproducible method for the stable transformation of tobacco protoplasts with Ti-plasmid DNA, using a similar selection procedure.

Protoplasts, isolated from leaves of aseptically grown *N. tabacum* SR₁ shoots, were cultured in K₃ medium supplemented

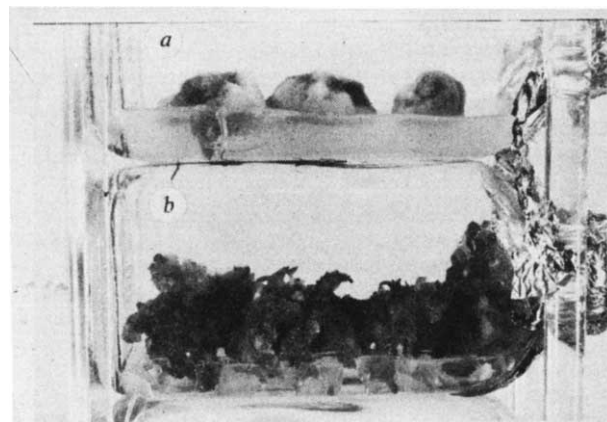


Fig. 1 Two callus lines obtained after *in vitro* DNA transformation growing in tissue culture on hormone-free LS medium. a, A typical non-regenerating line containing LpDH activity; pT₂-15. b, A typical line that regenerates shoots, but lacks LpDH activity; pT₂-6.

with phytohormones⁸ (see Table 1 legend). Normally, they form a new cell wall, divide and generate cell colonies (calli) which will eventually give rise to shoot-regenerating calli. In the present study transformants were selected on the basis of their phytohormone-independent growth. Other markers for the identification of transformants are the presence of lysopine dehydrogenase (LpDH) activity and T-DNA. Lysopine de-



Fig. 2 A mature, flowering plant obtained after grafting of a shoot derived from tissue line pT₂-2 on a stem of *N. tabacum* var. Samson⁹. Note the extended stamens of the flowers.

hydrogenase synthesizes octopine and related compounds such as lysopine in tumour cells induced by an *A. tumefaciens* strain carrying an octopine Ti-plasmid.

The present transformation experiments used octopine Ti-plasmid DNA from the wild-type strain Ach5(LBA4001). Plasmid DNA was dissolved at an appropriate concentration in sterile H₂O and its integrity and *Sma*I restriction pattern were checked by agarose gel electrophoresis. The DNA was stored in a sterile Eppendorf tube to which an equal volume of chloroform was added to kill any contaminants. The DNA was kept at 4 °C until use.

The transformation procedure involved incubating protoplasts and DNA in the presence of polyethylene glycol (PEG) and a post-incubation with high Ca²⁺ concentration (for details see Table 1 legend). This procedure is similar to one used in our laboratory for fusion of protoplasts to produce somatic hybrids. We first determined which PEG concentration would give sufficient cell survival (>20%) and negligible cell fusion during a 30-min incubation. In fusion conditions, PEG is usually present at a 40% concentration for several seconds. In the present experiments we found that a PEG concentration of 13.3% was optimal for an incubation of 30 min; cell survival was 50% and fusion <5%. In our initial DNA transformation experiments we determined whether it was best to start the post-incubation either with a one-step dilution of the PEG by adding 10 ml post-incubation mix containing 125 mM Ca²⁺ or gradually to dilute the PEG concentration stepwise while increasing the Ca²⁺ concentration, as is done in fusion experiments. We also examined whether the transformation was dependent on the concentration of Ti-plasmid DNA and the presence of 50 µg calf thymus (CT) DNA as a carrier. Out of 16 experiments, using different conditions, transformants were only found if 10 µg of Ti-plasmid DNA and 50 µg CT-DNA were used during the incubation and if the post-incubation mix was added stepwise. This procedure was tested twice (and both times gave rise to transformants) and Table 1 lists the transformed tissue lines obtained by this procedure in the two independent experiments. No phytohormone-independent calli were obtained in control experiments in which protoplasts were either incubated with CT-DNA alone or without addition of DNA.

From the first successful experiment (plasmid transformation 2 = pT₂), six tissue lines were obtained that showed both phytohormone-independent growth and LpDH activity, but no shoot regeneration. Five other lines proliferated without hormones but had no detectable LpDH activity (Fig. 1). These lines all had the capacity to regenerate shoots; two of them had initially shown the presence of the T-DNA coded enzyme, but had lost enzyme activity after 2 months of subculturing. When shoots that developed from these calli were placed on culture medium, they showed the same properties as regenerants obtained from

Table 1 Properties of tissues obtained after incubation of SR₁ protoplasts with pTi-Ach5 DNA

Tissue	Autotrophy	LpDH activity	Shoot regeneration
pT ₂ -2, 6, 8, 20*, 21*	+	—	+
pT ₂ -9, 10, 11, 13, 14, 15	+	+	—
pT ₂ -3	—	+	—
pT ₇ -2, 3	+	+	—

N. tabacum SR₁ protoplasts (pps) were isolated from sterile shoots and suspended in K₃ medium containing 0.4 M sucrose, supplemented with naphthaleneacetic acid (0.1 mg l⁻¹) and kinetin (0.2 mg l⁻¹) at a density of 5 × 10⁵ pps per ml (ref. 8). (All conditions used in the isolation of pps and the culture of SR₁ shoots are described in ref. 8.) Fractions (1 ml, that is, 5 × 10⁵ pps) were taken and 0.5 ml of polyethylene glycol (PEG 6000, 40% w/v) dissolved in a fusion medium¹¹ (F) containing 140 mM NaCl, 5 mM KCl, 0.75 mM Na₂HPO₄, 5 mM glucose and 125 mM CaCl₂·2H₂O pH 7.0 was added, followed by 10 µg of pTiAch5 DNA (0.4 mg ml⁻¹) and 50 µg of CT-DNA (1 mg ml⁻¹). Massive clumping of the protoplasts occurred. Protoplasts were incubated at 26 °C for 30 min with occasional shaking, after which 10 ml F-medium was added stepwise in 2-ml portions at 5-min intervals. Aggregates broke up during this post-incubation. The protoplasts were pelleted by centrifugation and the supernatant was removed. The pellet was resuspended in 10 ml of K₃ medium and plated in 10-cm Petri dishes after addition of carbenicillin (250 µg ml⁻¹). They were kept in the dark for 24 h and from then on in 2,000 lx for 12 h per day. Cell survival was ≥50%. Two weeks later, 5 ml of fresh K₃ medium was added. If the cell colonies were of sufficient size, they were plated in K₃ medium containing 0.3 M sucrose solidified with 0.3% agar and still supplemented with phytohormones. After ~1 month on this medium the small calli were placed for selection on hormone-free K₃ medium containing 0.2 M sucrose and 0.5% agar. Calli still growing after one or two passages on this medium were placed on hormone-free LS medium⁸. At this stage transformants were detected and tested for LpDH activity. pT₂ and pT₇ represent the only two independent experiments that have been performed with the described procedure, and both showed a positive result. Other conditions that were tested but gave negative results differed in incubation time, a lower Ca²⁺ concentration in the post-incubation, and plasmid DNA concentrations (1–50 µg) in the presence and absence of CT-DNA as a carrier. Other procedures were also used, including the poly-L-ornithine method and Ca²⁺ phosphate-DNA co-precipitation method, which in our hands gave negative results.

* These cell lines initially contained LpDH activity, but lost it after 2 months of subculturing.

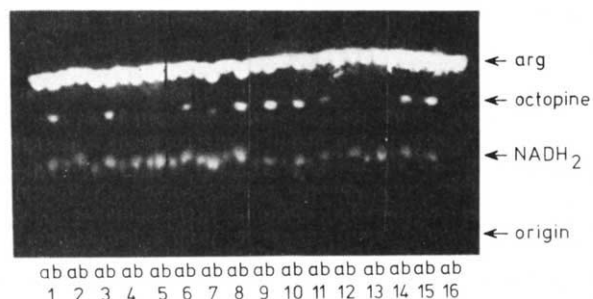


Fig. 3 Electropherogram of the products formed by LpDH activity in tissue extracts. The assay was performed as described by Otten¹². The reaction mixtures contained the extracts of the tissue lines listed in Table 1. *a*, Channels, reaction mixtures at *t* = 0; *b*, channels, reaction mixtures at *t* = 60 min; 1, B6S3 crown gall tumour callus (positive control); 2–13, pT₂ nos 2, 3, 6, 8, 9, 10, 11, 13, 14, 15, 20 and 21, respectively; 14, 15, pT₇ nos 2 and 3; 16, SR₁ shoot (negative control).

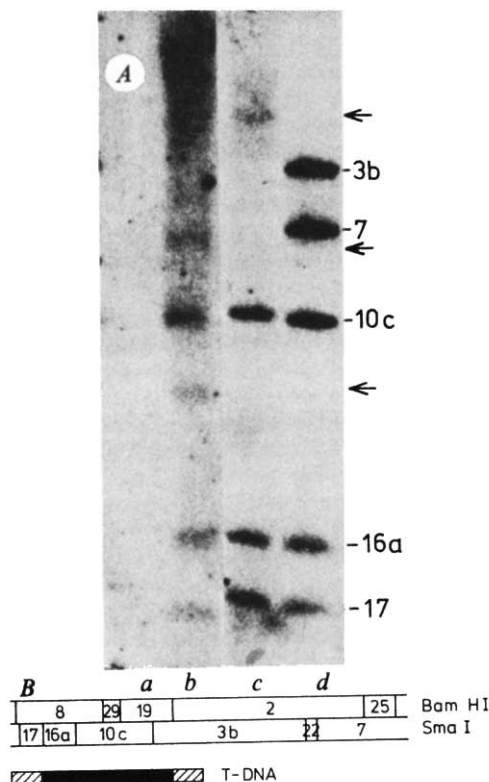


Fig. 4 A, detection of T-DNA in DNA transformed tissue line. The plant DNA was isolated as described by Heyn *et al.*¹³ and as modified by Chilton *et al.*¹⁴. The DNA was digested with the restriction endonuclease *Sma*I and fractionated by agarose (0.7%) gel electrophoresis¹⁰. Blots were prepared according to the procedure of Southern¹⁵ and hybridization was carried out as described by Thomashow¹⁶. A mixture of two recombinant DNA plasmids was used as DNA probe. They consisted of pBR322 in which *Bam*HI fragment 8 (pAL3252) and *Bam*HI fragment 29, 19, 2 and 25 (pAL3076) of the octopine Ti-plasmid were cloned, respectively. The plasmids were separately labelled *in vitro* with ³²P by nick translation¹⁷. The autoradiogram shows the hybridization of the probe to that of the plant DNA. Lane a, SR₁ (control); lane b, transformant pT₂-13; lane c, transformant pT₂-14; lane d, a one-copy reconstruction of the Ti-plasmid¹⁰. The arrows indicate the border fragments of the T-DNA in the transformants. B, a physical map of a portion of octopine Ti-plasmids representing the restriction endonuclease fragments of *Bam*HI and *Sma*I. The filled bar in the lower part of this figure shows the T-DNA organization as it is found in octopine crown gall tissues.

bacterial transformation experiments, that is, an inability to form roots and the absence of apical dominance, resulting in the development of shoots from lateral buds⁹. Several regenerants were grafted onto stems of *N. tabacum* var. Samson. Flowers of the mature, grafted shoots possessed an extended stamen and were male-sterile, as was observed previously for transformants⁹ obtained after bacterial infections of protoplasts (Fig. 2). Another line lost the ability for hormone-independent growth and is now being cultured on medium containing naphthaleneacetic acid and kinetin. It still had LpDH activity. A second independent experiment (PT₇) gave rise to two phytohormone-independent, non-regenerating tissue lines with LpDH activity. From the 14 transformed tissue lines obtained in two separate experiments, 11 have been stable from the beginning with regard to their phenotypic properties. The three lines which changed in phenotype have now been stable for at least 9 months. We do not know the reason for the variability in both number of transformants and their phenotype between the two experiments. The transformation event itself, using this procedure, is reproducible, but the transformation frequency and phenotypic diversity apparently differ between experiments. Figure 3 shows the result of the assay for LpDH activity in the various tissue lines.

As further evidence for the transformed nature of the tissues obtained, we have demonstrated the presence of Ti-plasmid

DNA sequences. DNA isolated from two tissue lines (phytohormone independent, LpDH⁺) was digested with the restriction endonuclease *Sma*I and fractionated by agarose gel electrophoresis. Southern blots were prepared from the gels and hybridized with *in vitro* labelled DNA probe. Figure 4 gives the results of hybridization for two lines, and shows the contiguous *Sma*I fragments 16a and 10c, which are always found in octopine tumour tissues and which are believed to carry sequences essential for the maintenance of the transformed state of the callus¹⁰; a band corresponding to *Sma*I fragment 17 is also present, located to the left of 16a; this has only been found in one cell line obtained at our laboratory by *in vivo* infection. Although both tissue lines analysed contain the same internal *Sma*I fragments, we conclude from the difference in border fragments in which plant DNA and bacterial DNA are joined (indicated by arrows in Fig. 4) that the two lines arose from independent transformation events within one experiment. The presence of *Sma*I fragment 17 in DNA transformants may indicate that frequently a larger part of Ti-plasmid is integrated into the host genome than on infection with the bacterium. Confirmation of this awaits further T-DNA analysis of other transformed tissue lines.

The larger extent of T-DNA in DNA transformants might indicate that the transformation of protoplasts is not due to an *A. tumefaciens* infection. The following arguments make the possibility of an infection leading to transformation unlikely. The experiments are performed in stringent aseptic conditions. The most conspicuous source of infection would be the plasmid DNA itself if bacteria had survived the isolation procedure. For this reason, the DNA was sterilized with chloroform and sterility was checked by plating a drop of the DNA solution on tryptone-yeast medium. No colonies were formed after 3 days at 29°C. Moreover, in our experience, *in vitro* transformation with *A. tumefaciens* only occurs in certain conditions (F. A. Krens *et al.*, in preparation): the protoplasts must be 3 days old, the incubation time must be >24 h and the density of the bacteria must be at least 3×10^5 per 10^5 protoplasts. None of these conditions is met in the procedure for DNA transformation.

The present results indicate that we have developed a reproducible method for the stable transformation of plant protoplasts with Ti-plasmid DNA. This, for the first time, conclusively demonstrates that the Ti-plasmid is the tumour-inducing principle and that *A. tumefaciens* itself is not a prerequisite for obtaining tumour cells. DNA transformation experiments may aid in elucidating the molecular basis of crown gall tumorigenesis. Experiments have already been started using mutated Ti-plasmids carrying insertions that lead to avirulence of the mutants *in vivo*. The evidence presented here that plant cells can be stably transformed with DNA will undoubtedly also stimulate programmes for genetic engineering in higher plants.

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A viral polymerase involved in recognition of influenza virus-infected cells by a cytotoxic T-cell clone

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Influenza virus-specific cytotoxic T lymphocyte (CTLs) can be obtained from mice and humans infected with influenza A viruses^{1,2} and appear to be beneficial in the host response³. The viral target structure recognized by influenza virus-specific CTLs remains a controversial question for two reasons: first, previous specificity analyses were difficult to interpret because of heterogeneous CTL populations, and second, more than one viral protein has been detected serologically on the surface of influenza-infected cells⁴⁻¹⁰. To avoid these problems, anti-influenza A/PR/8/34 CTL clones were generated and tested for cytolytic activity on target cells infected with recombinant viruses containing known rearrangements of the genes of strains A/PR/8/34 and A/HK/1/68. We report here that one such CTL line lysed target cells only if the infecting virus possessed the polymerase gene P3 of A/PR/8/34. This suggests that the P3 gene product either induces a virus-specific cell-surface antigenic modification or is expressed on the surface of the infected cell, and hence is involved in recognition of target cells by the anti-influenza CTL line.

Influenza type A-specific CTLs were generated as described elsewhere^{11,12}. BALB/c mice were inoculated intraperitoneally (i.p.) with A/PR/8/34; 2-4 weeks later, the primed spleen cells were cultured *in vitro* for 10 days in the presence of PR8-infected BALB/c spleen cells. Our method differs from the standard technique in that the culture from which the CTL line described here (BCIP3.1) was derived was then treated with monoclonal anti-Lyt 2 antibody 3-168.8 (ref. 13) (the gift of F. Fitch, University of Chicago) plus complement. Initially this treatment was performed to enrich for helper T cells. Although the percentage of Lyt 2⁺ cells was reduced at the time of treatment to less than 1% (as shown by immunofluorescence), most of the cultures retained antiviral CTL activity and ~5-10% were 'strain-specific'. CTL clones derived from these cultures have subsequently been shown to be Lyt 2⁺ by immunofluorescence. 'Cross-reactive' CTL lines alone were isolated from cultures not treated with anti-Lyt 2 antibodies. All cultures were propagated *in vitro* in medium containing 20% supernatant from concanavalin A (Con A)-stimulated rat spleen cell cultures and, after 2-3 months, cloned on a feeder layer of irradiated BALB/c peritoneal exudate cells. The CTL line BCIP3.1 described here has been maintained in culture for 6 months.

The specificity of the BCIP3.1 line was examined in micro-cytotoxicity assays on P815 cells infected with various influenza viruses. As shown in Table 1, the cytotoxic activity was restricted to cells infected with influenza viruses A/Swine/31 (A/SW/31), A/Puerto Rico/8/34 (A/PR/8/34), A/Melbourne/35 (A/Mel/35) and A/BH/35. The failure of BCIP3.1 to lyse target cells infected with the other influenza A viruses was not due to inadequate expression of viral antigens on these target cells as they were killed by one or several other CTL lines included as positive controls (J.R.B.; in preparation).

As the BCIP3.1 clone lysed cells infected with A/Puerto Rico/8/34 (PR8) but not with A/Hong Kong/1/68 (HK) (Table 1), it was possible to examine its specificity further using target cells infected with recombinant viruses containing various

arrangements of parental PR8 and HK genes. Due to the segmented genome of influenza viruses, such recombinant viruses can be readily produced by mixed infection, and their genotype determined by RNA-gel electrophoresis¹⁴. Thus, the BCIP3.1 line was tested for cytotoxic activity against P815 cells infected with 17 different PR8-HK recombinant viruses of defined genotypes (gifts of Drs P. Palese, J. Schulman and M. Lubeck, Mt Sinai School of Medicine). It can be seen from the top half of Table 2 that the BCIP3.1 clone does not lyse targets infected with 10 recombinant viruses which lack the PR8 P3 gene. Particularly striking is the recombinant E61-24-P15 which differs from PR8 solely in the substitution of the P3 gene. This absence of cytotoxicity is due neither to the failure of these viruses to infect the target cells, nor to a general resistance of the infected cells to lysis by CTLs, as the cells were lysed to the same extent as PR8-infected cells by cloned CTL lines specific for other viral target structures (J.R.B., in preparation). In contrast, the bottom half of Table 2 shows that target cells infected with recombinant viruses having the PR8 P3 gene were lysed. As PR8 P3 is the only gene in the recombinant virus panel which correlates absolutely with the presence or absence of cytotoxic activity, we conclude that the BCIP3.1 T-cell clone recognizes a target structure on P815-infected cells which requires the presence of the PR8-derived P3 gene.

However, lysis of recombinant virus-infected target cells by the BCIP3.1 clone is not a simple all-or-none phenomenon because the extent of lysis seems to be affected to some extent by additional genes: for example, the highest ⁵¹Cr release (45-60%) was observed for the four recombinants E61-24-H16, J1, 13B and J5 which have in common the PR8-derived genes P3 (prerequisite for lysis), P2 and Np. However, substitution of either the P2 or nucleoprotein (NP) genes of PR8 by the corresponding HK genes resulted consistently in reduced ⁵¹Cr

Table 1 Cytotoxic activity of BCIP3.1 CTL line on P815 cells infected with various influenza virus strains

Virus strain (subtype)	% Specific ⁵¹ Cr release
A/Swine/31	27
A/WSN/33	3
A/Puerto Rico/8/34	37
A/Melbourne/35	31
A/BH/35	17
A/Hickox/40	2
A/Bellamy/42	1
A/Weiss/43	5
A/Cameron/46	0
A/Fort Monmouth/1/47	0
A/Forth Worth/50	2
A/Malaya/54	0
A/Denver/57	0
A/Ann Arbor/57	0
A/Japan/305/57	0
A/Hong Kong/1/68	0
A/Victoria/68	0

Type A influenza viruses have been grouped into three subtypes designated H1N1, H2N2 and H3N2. H1, H2 and H3, and N1, N2 each designate serologically non-cross-reactive serotypes of the haemagglutinin (HA) and neuraminidase (NA) molecules, respectively. Each subtype consists of many distinct but antigenically related glycoproteins that characterize individual virus strains. Infectious allantoic fluid of these viruses was used to infect P815 cells as described previously⁵. Six hours after infection, cells were incubated for 1 h with ⁵¹Cr at 37 °C, washed twice and then dispensed into wells of microtitre plates (10⁴ cells per well). BCIP3.1 cell ratio of 9:1. The cells were pelleted lightly and after 4 h incubation at 37 °C, one-half of the culture supernatant was removed for determination of the concentration of released ⁵¹Cr by γ counting. Per cent specific release was determined according to the equation: experimental release (in the presence of CTLs) - spontaneous release (no CTLs)/total release (in the presence of detergent) - spontaneous release. Each assay was performed in triplicate.

release, that is, 23–26% and 13% for recombinants E61-13-H17, E61-13-H20 and E61-13-H19 respectively.

The frequency of CTLs possessing the BCIP3.1 specificity in the influenza immune response is unknown. Previous frequency analyses could have placed this specificity with either 'strain-specific' or 'cross-reactive' CTLs, depending on the viruses tested. The finding of at least two other clones, one having an apparently identical specificity and another with a slightly different specificity but correlating with the polymerase gene complex (unpublished data) suggests that the BCIP3.1 is not an unusual clone. The role and relative importance of the polymerase gene complex in the CTL immune response are unknown.

The association of the lytic activity of BCIP3.1 with the presence of the PR8 P3 gene can be explained by two mechanisms. First, BCIP3.1 could recognize the P3 protein itself. Although P3 is present in infected cells in only small quantities compared with the other viral proteins, and appears to be located predominantly in the nucleus and cytoplasm^{15,16}, it remains a possible target structure, as the amount of viral antigen needed for lysis by CTLs is unknown and the possible presence of small amounts of cell-surface-associated P3 has not been investigated. Alternatively, P3 may be required for the expression of the target structure recognized by BCIP3.1. In this respect, note that the function of the P3 protein in influenza virus infection is incompletely understood. Results obtained for temperature-sensitive mutants indicate a role for P3 in complementary (messenger) RNA synthesis¹⁷, and suggest that P3 is involved in the transport of the viral transcription–replication complex (consisting of the P1, P2, P3 and NP proteins¹⁶) from nucleus to cytoplasm¹⁸. Thus the PR8 P3 protein may modify another viral (or cellular) gene product recognized by the BCIP3.1 line, or may be responsible for transport of this antigen to the cell surface. The finding that the cytotoxic activity of BCIP3.1 is also related to the presence of two other proteins (P2 and NP) known to be involved in the virus transcription–replication complex, does not help to distinguish between these possibilities as this observation could reflect either the involvement of P2 and NP in the surface expression of P3 or the higher efficiency of a PR8 P2–P3–NP complex in the expression of the actual antigen recognized by BCIP3.1.

Table 2 Cytotoxic activity of BCIP3.1 CTL line on P815 cells infected with various recombinant influenza viruses

Virus	Genotype								% Specific ⁵¹ Cr release
	P1	P2	P3	HA	NA	NP	M	NS	
PR8	P	P	P	P	P	P	P	P	21–47
HK	H	H	H	H	H	H	H	H	2–7
E61-24-P15	P	P	H	P	P	P	P	P	1
E61-13-P15	H	H	H	P	P	P	P	P	0
E61-24-P11	P	P	H	P	P	P	P	H	0
P37	H	P	H	P	H	P	P	P	0
E61-13-H15	H	P	H	H	H	P	H	P	1
E61-13-P19	H	H	H	P	P	H	H	P	0–4
3385	H	H	H	H	H	P	P	P	4
3376	H	H	H	P	H	H	P	H	2
E61	H	H	H	P	P	H	H	H	1
P50	H	H	H	P	H	H	H	H	6
E61-24-H16	H	P	P	H	H	P	P	H	60
J1	P	P	P	H	P	P	P	P	47
13B	P	P	P	H	P	P	H	P	45
J5	H	P	P	H	P	P	H	H	51
E61-13-H17	P	P	P	H	H	H	P	P	26
E61-13-H20	H	P	P	H	H	H	P	P	23
E61-13-H19	H	H	P	H	H	P	H	P	13

⁵¹Cr-release assays were performed on P815 cells 7 h after infection with recombinant viruses containing various combinations of PR8 (P) and HK (H) genes coding for the three viral polymerases (P1, P2 and P3), haemagglutinin (HA), neuraminidase (NA), nucleoprotein (NP), matrix (M) and non-structural (NS) proteins. The pooled data from three separate cytotoxicity assays are shown.

In conclusion, we have demonstrated that the PR8 P3 gene product, perhaps in association with other viral gene products, is involved in the recognition of target cells by an anti-influenza CTL line. However, it remains to be determined how P3 affects the target cell surface and whether it plays an important part in the recognition of influenza-infected target cells by CTLs.

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Monoclonal antibody to Lyt 2 antigen blocks H-2I- and H-2K-specific mouse cytotoxic T cells

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It has been proposed that the murine cell surface antigen Lyt 2 could serve to distinguish cytotoxic T lymphocytes (CTLs) and their immediate precursors, which bear Lyt 2, from helper T cells, which do not¹. Later work, however, has demonstrated that Lyt 2⁺ cells can 'help' B cells mature into antibody-secreting cells, provided that the B cells differ from the allo-helper T cells at the K or D region of the H-2 major histocompatibility complex (MHC)². Furthermore, a T-cell line has recently been described which is Lyt 2⁺ but specifically cytotoxic for H-2I region determinants^{3,4}. These new findings have prompted an alternative hypothesis, that Lyt 2 antigen does not discriminate between cytotoxic and non-cytotoxic (helper) T cells, but rather between I-region- and K/D-region-specific cells⁵. In this new view, the apparent association between presence of Lyt 2 antigen and killer T-cell function merely reflects the tendency, by no means absolute^{6–8}, for CTLs to recognize K- or D- rather than I-region determinants⁹. We show here that I-region-specific murine CTLs, generated in primary *in vitro* culture, do in fact bear the Lyt 2 antigen, as do CTLs directed against the K region of H-2.

Our method is based on the observation that the lytic reaction between mouse CTL effectors and their targets can be blocked by addition of anti-Lyt 2 antibody directly to the cytotoxicity assay^{10–13}. Although it is not clear how the antibody interferes with target cell lysis, the finding that inhibition does not require Lyt 2 expression by the target cells strongly suggests that recognition of the Lyt 2 molecules on the CTL effector cell is

Table 1 Cytolysis by *I*-region-specific CTLs is inhibited by monoclonal antibody to Lyt 2

	Line	Responder	Stimulator	Target	Antigen	Antibody added	% Specific lysis (s.e.m.)
Expt A	1	AQR	T6R	T6R	I ^a	None	66 (2)
	2	AQR	T6R	T6R	I ^a	Anti-Lyt 2	3 (2)
	3	AQR	A	A	K ^k	None	53 (3)
	4	AQR	A	A	K ^k	Anti-Lyt 2	0 (1)
Expt B	5	AQR	T6R	T6R	I ^a	None	26 (3)
	6	AQR	T6R	T6R	I ^a	Anti-Lyt 2	4 (1)
	7	AQR	A	A	K ^k	None	14 (3)
	8	AQR	A	A	K ^k	Anti-Lyt 2	2 (1)
	9	T6R	AQR	AQR	I ^k	None	11 (1)
	10	T6R	AQR	AQR	I ^k	Anti-Lyt 2	3 (1)
Expt C	11	AQR	T6R	T6R	I ^a	None	34 (3)
	12	AQR	T6R	T6R	I ^a	Anti-Lyt 2	12 (1)
	13	AQR	T6R	T6R	I ^a	Anti-Lyt 2.2	9 (1)
	14	AQR	T6R	T6R	I ^a	Nude NMS	33 (3)
	15	AQR	T6R	T6R	I ^a	Anti-Lyt 1	50 (4)
	16	AQR	T6R	AQR	None	None	2 (1)
	17	AQR	A	A	K ^k	None	48 (5)
	18	AQR	A	A	K ^k	Anti-Lyt 2	5 (2)
	19	T6R	AQR	AQR	I ^k	None	28 (3)
	20	T6R	AQR	AQR	I ^k	Anti-Lyt 2	7 (1)
	21	T6R	AQR	T6R	None	None	2 (1)

Spleen cells from unprimed responder mice were mixed with irradiated (1,500 rad) stimulator spleen cells, and cultured in conical-bottomed microtitre wells such that each well contained 3×10^5 responder cells and 5×10^5 stimulators in 0.2 ml of RPMI-1640 medium with 10% fetal calf serum, 10 mM HEPES, antibiotics and added glutamine. After 5 day culture at 37 °C, the medium was shaken off and the cell pellet resuspended in 0.1 ml of serum-supplemented medium containing the antibody to be tested for inhibitory effect. Another 0.1 ml of medium was then added containing 2×10^4 ^{51}Cr -labelled concanavalin A-stimulated spleen cells of the indicated strain, as targets. After a 4 h incubation at 37 °C, the plates were centrifuged at 1,000 r.p.m. for 10 min and 0.1 ml of supernatant was removed for counting. Per cent specific lysis was calculated as $100 \times (\text{experimental} - \text{low control}) / (\text{acid lysis} - \text{low control})$. Six to eight statistically independent replicates were performed for each experimental condition tested; the equivalent ratio of viable effector cells to targets is ~10:1. Anti-Lyt 2 is hybridoma clone 53-6.7 of ref. 15, used at 1/100 in expt A, and 1/500 in expts B and C. Normal nude mouse serum (nude NMS) and anti-Lyt 1 antibody (clone 53-7.3)¹⁵ were used at 1/500. Ascites fluid containing mouse monoclonal antibody to Lyt 2.2 was used at 1/100. Strain abbreviations and genotypes are as follows: AQR, B10.AQR($K^a I^k D^d$); T6R, B10.T(6R) ($K^a I^a D^d$); A, B10.A($K^k I^k D^d$). All three strains are Tla^a.

involved^{10,11}. Cytotoxicity can be blocked by conventional alloantisera and by monoclonal antibodies specific for the Lyt 2 molecule¹⁰⁻¹³.

To examine *I*-region-specific CTL responses of unprimed mice, which have been reported to be considerably weaker than the *in vitro* responses obtained from *in vivo* immunized responders⁶, we performed the 5 day *in vitro* sensitization culture and the 4 h ^{51}Cr -release assay for cytotoxicity in conical-bottomed microtitre wells, which we have found produce satisfactory responses to several 'weak' antigens (that is, non-H-2 determinants). To generate *I*-region immune CTL effectors, we carried out reciprocal *in vitro* sensitizations between congenic-resistant mouse strains B10.AQR and B10.T(6R), which differ only at the *I* region of the MHC¹⁴. B10.A stimulators, which differ from B10.AQR only at the *K* region, were used to produce *K*-specific CTLs as a positive control. At the end of the 5 day sensitization period, the CTL assay was carried out in the presence of either monoclonal antibody to Lyt 2 or control serum. A monoclonal rat antibody to a nonpolymorphic portion of the murine Lyt 2 molecule was used (clone 53-6.7 of ref. 15), and we found that this antiserum inhibits both *I*- and *K*-region-specific CTL reactions at dilutions $>1/3,200$.

Table 1 shows the results of three consecutive experiments; the monoclonal antibody to Lyt 2 not only blocks *K*-specific CTLs as expected, but also interferes with *I*-region-specific CTLs, and to the same extent. CTLs specific for I^a (Table 1, lines 2, 6, 12), I^k (Table 1, lines 10, 20) and K^k (Table 1, lines 4, 8, 18) are all strongly inhibited by the addition of anti-Lyt 2 antibody. The specificity controls show that the CTLs are in fact *I*-region specific (compare lines 11, 16 and 19, 21), as they kill only those targets which present the appropriate alloantigen; target cell death thus cannot be attributed to an antigen-nonspecific cytolytic event. Because the source of monoclonal antibody was serum from nude mice bearing the hybridoma as a subcutaneous tumour, controls were carried out (experiment C of Table 1) to show that the inhibitory activity was attributable to the anti-Lyt 2 antibody in the serum, rather than to some unsuspected material which might be present in sera from tumour-bearing nude mice. In studies involving conventional (non-monoclonal) antibodies of the Lyt type, the best control¹⁶ is to compare the

effect of the antibody on responder mice which differ only at the locus in question (here Lyt 2), but such a control is impossible in our study because it would require mice that are congenic at both the Lyt 2 and *I*-region loci. Table 1 shows that neither normal nude mouse serum (Table 1, line 14) nor serum from nudes carrying a hybridoma (clone 53-7.3 of ref. 15), which secretes antibody to mouse Lyt 1 antigen (Table 1, line 15), was able to inhibit CTL effector activity, showing that the inhibitory activity of the anti-Lyt 2 hybridoma antiserum depended on the presence of antibodies to Lyt 2. Furthermore, line 13 in Table 1 shows that strong inhibition of cytotoxicity can also be obtained by a second monoclonal anti-Lyt 2 antiserum (provided by Dr U. Hammerling), which is specific for the polymorphic determinant Lyt 2.2 and is used as ascitic fluid from tumour-bearing mice. As inhibitory activity is found only in those sera from hybridoma-bearing nude mice which include anti-Lyt 2 antibody, and both monoclonal reagents (reactive with different determinants on the Lyt 2 molecule) exhibit potent blocking activity, we are confident that the inhibition seen results from an interaction between anti-Lyt 2 antibody and CTL surface Lyt 2 molecules. In summary, Table 1 shows that CTL activity against both *I*- and *K*-region antigenic determinants can be blocked by monoclonal anti-Lyt 2 antibodies, and that this blocking is attributable to the anti-Lyt 2 antibody in such reagents.

Although inhibition of lysis by anti-Lyt 2 antibody, as argued above^{10,11}, need not involve Lyt 2-bearing target cells, we considered whether the inhibition shown in Table 1 might involve, not an interaction between the antibody and the CTL effectors, but rather a previously unknown protective effect of anti-Lyt 2 antibody on the concanavalin A blast targets, a portion of which exhibit Lyt 2 antigen¹². The experiment shown in Table 2, however, shows that strong inhibition of cytotoxicity by anti-Lyt 2 antibody is obtained even when the target cells are lipopolysaccharide activated B cells, selected by adherence to plastic plates coated with anti-mouse immunoglobulin; these cells do not carry surface Lyt 2. Thus, inhibition of *I*-region-specific CTLs can be induced by an interaction between anti-Lyt 2 antibody and the CTL effector cell itself.

It is not yet clear at what stage of cell-mediated cytotoxicity the Lyt 2-mediated blocking is achieved, although the

Table 2 Inhibition of *I*-region-specific CTLs by anti-Lyt 2—B cell blast targets

Responder	Stimulator	Antibody added	% Specific lysis (s.e.m.)
AQR	T6R	None	45 (2)
		Anti-Lyt 2	17 (5)
		Anti-Lyt 1	41 (3)
		Nude NMS	43 (2)
T6R	AQR	None	54 (4)
		Anti-Lyt 2	28 (3)
		Anti-Lyt 1	52 (4)
		Nude NMS	45 (3)

Experimental conditions were as for Table 1, except that the ^{51}Cr -labelled targets were plate-selected²⁶ B cells stimulated with lipopolysaccharide ($5\text{ }\mu\text{g ml}^{-1}$) for 48 h before use. All inhibitory antibodies were used at 1/300 dilution. Per cent lysis is presented as mean (and s.e.m.) of 16 wells per group.

tantalizing proximity of the Lyt 2 gene to the mouse V_{κ} locus¹⁷, which controls the variable region of the κ light chain, and the demonstration¹³ that anti-Lyt 2 antibody blocks conjugate formation, but not lysis itself, suggest that inhibition of specific target recognition may be involved. In any case, our results indicate that however the Lyt 2-bearing molecules in the effector cells are involved in cytotoxicity, they are likely to have an equivalent role regardless of the genetic region—*I* or *K/D*—which controls the target cell antigens being recognized by the effector CTL. They also demonstrate, *a fortiori*, that *I*-specific CTLs induced by primary *in vitro* sensitization must be Lyt 2⁺ cells.

We cannot exclude the possibility that some *I*-specific CTLs produced from normal spleen cells might be Lyt 2⁺, and thus resemble the Lyt 2⁺, *I*-A^k-specific cytolytic T-cell line recently described^{3,4}, but we believe that most *I*-specific CTLs, like their more easily generated *K/D*-specific counterparts¹ and many cytolytic T-cell lines^{18,19}, are probably Lyt 2⁺, based on the unambiguous inhibition of cytotoxicity produced by the anti-Lyt 2 reagents in the present experiments. Our results are consistent with a recent study²⁰ showing that *I*-specific primary CTL effectors could be killed by sufficiently potent anti-Lyt 2 antisera and complement.

Generation of cytotoxic effector cells is now believed to involve collaboration between the CTL precursors and a 'helper' T cell which produces the helper factor interleukin-2 (IL-2)^{21,22}. Although *K*-region-stimulated secretion of IL-2 by Lyt 1⁺2⁺ cells has been reported recently^{23,24}, we have found²⁵ that positively selected Lyt 2⁺ cells produce much less IL-2, even in response to *K*-region disparities, than do *I*-region-stimulated Lyt 2⁺ cells. We thus favour a model for CTL generation across MHC incompatibilities in which most of the IL-2-producing helper cells are *I*-region sensitive and Lyt 2⁺ and most of the CTLs, whether *K*- or *I*-region responsive, are nonetheless Lyt 2⁺. The extent to which the allospecific CTLs resemble CTLs generated *in vivo* or to 'foreign' antigens in syngeneic contexts requires further investigation.

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Glycolipid affinity purification of migration inhibitory factor

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Migration inhibitory factor (MIF) is an immune mediator secreted by primed lymphocytes on *in vitro* challenge by antigen or mitogen, which elicits various physiological responses from its target cell, the macrophage^{1,2}. Previous studies suggest that membrane glycoconjugates have an important role in regulating the interaction between MIF and the macrophage. A macrophage glycolipid functions as a putative receptor for MIF as evidenced by its ability to enhance the macrophage response to MIF^{3,4}. This enhancing ability of glycolipids requires the presence of sialic acid⁵ and fucose⁶; these carbohydrate residues are also required on the macrophage surface for the induction of an intact response by MIF^{5,7}. Furthermore, it has been reported⁴ that incubating a preparation of mixed gangliosides from bovine brain with lymphocyte supernatants abolished their MIF and macrophage activating factor (MAF) activities. Taken together, these results are evidence that a glycolipid serves as a membrane receptor for MIF. We predict that glycolipids which enhance the macrophage response to MIF, also bind MIF with subsequent elution of biological activity, and present here evidence in support of this notion. We also demonstrate that glycolipid covalently bound to agarose provides a novel means of affinity purification of MIF.

Guinea pig⁸ and human⁹ MIF have been fractionated in this laboratory by various conventional techniques including gel filtration, electrophoresis and isoelectrofocusing. These procedures separate macromolecules on the basis of their physicochemical properties and thus are limited by their lack of specificity. Affinity chromatography is a useful technique for the purification of many proteins, and has been used successfully to purify MIF^{10,11}. Here we used an insoluble agarose support, derivatized with a macromolecular arm (poly-L-lysine¹²) followed by covalent attachment of gangliosides to this support¹³, to purify guinea pig MIF.

Results of initial purification experiments are shown in Fig. 1. MIF does not bind or is not adsorbed to columns containing unsubstituted agarose in the presence of phosphate-buffered saline (PBS) (0.15 M NaCl in 0.01 M phosphate-buffer pH 7.4). However, a substantial amount of MIF activity does adsorb to agarose covalently bound to a mixed preparation of bovine brain gangliosides. This adsorption appears to be specific as no adsorption occurs on agarose substituted with other preparations of glycolipids, for example, disialoganglioside (GD_{1a}), bovine brain grey matter and guinea pig brain (Fig. 1). Furthermore, the adsorbed MIF activity was recovered by elution with 0.2 M fucose in 1 M potassium thiocyanate (KSCN). Recovery of active MIF showed that use of ganglioside-agarose to purify MIF was possible.

In the experimental protocol described in Fig. 1 legend, a significant amount of protein (12–30%) eluted with MIF (data

Table 1 Ability of glycolipids to enhance MIF response

Macrophages preincubated with:		Bovine brain mixed gangliosides	% Inhibition of migration Guinea pig macrophage glycolipid	Bovine brain grey matter glycolipid	Guinea pig brain glycolipid
Expt					
1	6	37		1	
2	3	38		3	
3	6	30		0	
4	20	48		0	
5	7		36		22
6	28		43		24
7	24		35		17
8	24		52		4

Glycolipid preparations were tested for their ability to enhance the macrophage response to MIF, using methods described elsewhere³. Briefly, the test glycolipid deposited with lecithin and cholesterol as a thin film on a glass wall *in vacuo*. Liposomes were formed under nitrogen in buffer by agitation and sonication. After centrifugation the small liposomes were incubated with macrophages at 37 °C for 1 h. Cells were then washed and assayed for their response to a low dose of MIF in a capillary tube assay. The MIF response is shown as % inhibition of migration. The preparation of mixed gangliosides from bovine brain and glycolipids from guinea pig macrophages were the only preparations able to enhance the macrophage response to MIF.

not shown). Experiments were done to investigate whether different buffer conditions would remove unwanted protein from the eluent (Fig. 2). MIF was allowed to bind in a buffer of relatively high salt concentration (0.3 M NaCl in 0.01 M phosphate buffer pH 7.4 [0.3 M PBS]). The column was then washed with 0.6 M NaCl in 0.01 M phosphate buffer pH 7.4 (0.6 M PBS) and MIF eluted with 1.0 M KSCN in 0.01 M phosphate buffer pH 7.4 (1.0 M PBS). Most of the protein eluted before MIF (Fig. 2), thereby facilitating a significant increase in the specific activity of MIF (Table 2).

The results shown in Table 1 indicate a correlation between the reversible binding activity of a glycolipid preparation and its ability to enhance the macrophage response to MIF. We succeeded in enhancing MIF activity by preincubating macrophages with their putative glycolipid receptors for MIF as described previously⁶. The only glycolipid preparation to which MIF could be bound and recovered (mixed ganglioside from bovine brain) was also able to enhance the macrophage response to MIF—further evidence that gangliosides serve as MIF receptors.

As carbohydrate is known to be an essential part of the macrophage response to MIF⁵⁻⁷ we investigated the use of fucose as a specific eluent in a more effective purification method for MIF. Preliminary studies indicate that fucose alone is unable to elute MIF from ganglioside-agarose columns. KSCN alone was effective in the recovery of MIF, which is not surprising in view of what is known about carbohydrate-protein interactions. Simple monosaccharides, such as fucose, exhibit biological effects only at very high concentrations^{7,10,14}. In the case of ganglioside receptors, it appears that the specific oligosaccharide sequence and not just the presence of sialic acid is important for receptor function^{15,16}. It may be necessary to elute MIF with the oligosaccharide moiety of the putative glycolipid receptor. This could also explain why the ganglioside-agarose matrix is still effective for the purification of MIF even though the gangliosides are covalently bound to the polylysyl backbone

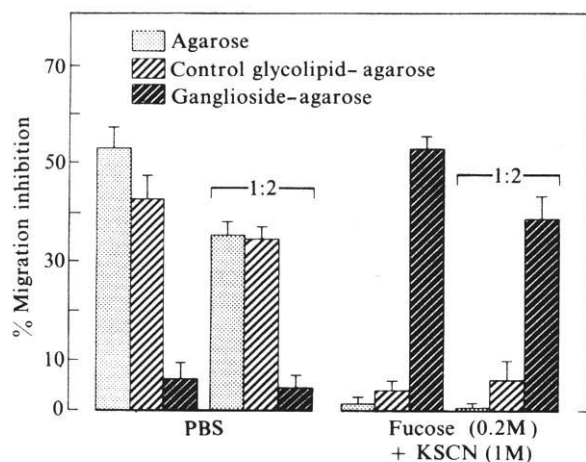


Fig. 1 Specific binding of MIF to ganglioside-agarose columns. Guinea pig MIF was prepared from culture supernatants of concanavalin A-stimulated lymph node cells from PPD-sensitized Hartley guinea pigs and chromatographed on Sephadex G-100 columns. One unit of MIF was defined as the greatest dilution giving 20% inhibition of migration in the capillary tube assay⁵. To determine the number of MIF units in a particular fraction, serial twofold dilutions were assayed. Sepharose 4B (Pharmacia) was activated by CNBr and coupled to poly-L-lysine (molecular weight 500,000; Sigma)¹². Mixed gangliosides from bovine brain (Supelco) were coupled to poly-L-lysine-agarose using carbodiimide¹³. Approximately 80% of the gangliosides were insolubilized as determined by the amount of free sphingosine in the reaction mixture. The coupled agarose was diluted fivefold with Sepharose 4B and packed in glass columns (1 × 10 cm) with a total bed volume of 3–4 ml. MIF (16–20 units or ~10 ml equivalents of original supernatant) was percolated on to the column at room temperature with a flow rate not exceeding 10 ml h⁻¹. The column was equilibrated and washed with PBS buffer followed by 0.2 M fucose in 1 M KSCN; 10 ml of each wash were collected on ice, vacuum-concentrated to 3 ml and dialysed overnight against minimal essential medium plus Earle's salts (Microbiological Associates). The equilibration and elution buffers and the types of agarose used are indicated. □ represents unsubstituted agarose (poly-L-lysine-agarose without ganglioside); ▨ represents agarose coupled to glycolipids prepared from bovine brain grey matter⁶, guinea pig whole brain⁶ or GD_{1a}; ▩ represents agarose coupled to bovine brain mixed gangliosides (BGSL). Note that MIF activity (undiluted and 1:2 dilution) elutes through the control columns with the PBS wash and through the BGSL-agarose column only with the fucose-KSCN buffer. The differences between all six experiments were statistically significant ($P < 0.001$) by Student's *t*-test.

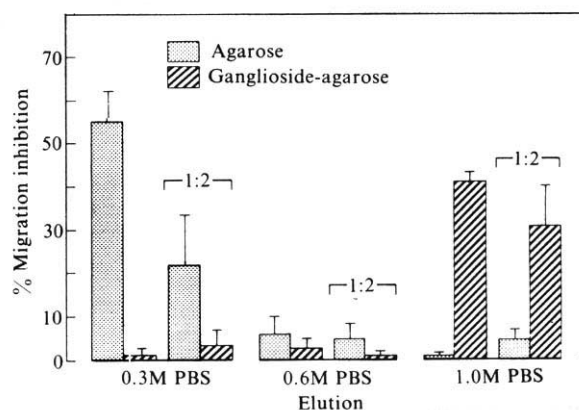


Fig. 2 Purification of MIF by salt elution from ganglioside-agarose columns. Partially purified MIF was subjected to affinity chromatography on ganglioside-agarose columns as described in Fig. 1 legend with the following modification. The equilibration and two sequential buffer elutions consisted of 0.01 M phosphate buffer pH 7.4 with 0.3 M NaCl, 0.6 M NaCl, and 1.0 M KSCN, respectively. □ represents unsubstituted agarose; ▩ represent agarose coupled to bovine brain mixed ganglioside. Note that MIF activity could be quantitatively recovered in the 1.0 M KSCN elution after binding in 0.3 M NaCl buffer. In three experiments, differences between each of the elutions were statistically significant ($P < 0.001$).

Table 2 Affinity purification of MIF on ganglioside-agarose

	Total protein (mg)	Total units	Specific activity (U mg ⁻¹)	Purification
Before purification	0.20	16	80	1
0.3 M	0.061	0	0	0
0.6 M	0.016	0	0	0
1.0 M	0.0017 (3%)	13.4 (84%)	1,914	24

The data summarize an experiment described in Fig. 2 legend. Percentage yield is given in parentheses. The breakthrough is represented by 0.3 M PBS, elution 1 is represented by 0.6 M PBS and elution 2, 1.0 M PBS. Purification may be more or less marked depending on the MIF preparation. One unit of MIF activity was defined as the highest dilution giving 20% inhibition of migration in the capillary tube assay.

through their sialic acid residues. MIF may recognize the oligosaccharide and not just sialic acid.

However, the method used here for coupling gangliosides to agarose does present the possibility that hydrophobic forces are involved in the ganglioside-MIF interaction, together with an affinity of MIF for carbohydrate residues. The dual nature of interactions between macromolecules has been shown previously for interferon¹⁴ and tetanus toxin¹⁷. Furthermore, the increasing ionic strengths of the buffers used in the protocol described in Fig. 2 legend may strengthen the hydrophobic interactions. This would result in a high binding affinity necessitating the use of chaotropic ions, such as thiocyanate in the case of MIF and serum growth factor¹⁸ or 7 M guanidine HCl in the case of cholera toxin¹³, which promote macromolecular unfolding and dissociation.

It is probable that only a minor unknown component of the bovine brain ganglioside mixture has a biospecific affinity for MIF⁴. We therefore have to consider the overlapping effects of other ganglioside-protein interactions which obscure the particular interaction in question. It might be better to examine an affinity adsorbent that contains only the homogeneous putative glycolipid receptor for MIF¹⁹.

We have shown that gangliosides covalently bound to derivatized agarose provide a powerful tool for the purification of MIF by solid phase affinity chromatography. Furthermore, it may provide a means of separating biochemically similar lymphocyte mediators. Work is being done to define further the nature of the binding between the gangliosides and MIF components.

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Persistence of axial orientation cues in regenerating intima of cultured aortic explants

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The endothelial cells of arteries have a preferential orientation parallel to the axis of blood flow¹. This axial orientation is maintained even at the interface between two branching or anastomosing vessels¹, and is re-established by newly regenerated endothelial cells in regions of intimal wounds². The cause of the orientation, however, remains unclear. Two possibilities have been proposed, shear stress from blood flow³ and 'contact guidance' by extracellular matrix⁴, but experiments to distinguish between them have been limited by the inaccessibility of the intima to systematic manipulation *in vivo*, and by the failure of organ-cultured vessels to demonstrate intimal regeneration comparable with that observed *in vivo*^{5,6}. We have recently developed a technique for the culture of whole rat aorta explants which provides easy access to the intimal surface *in vitro*⁷. Additionally, our technique provides the first organ culture system in which the regeneration of endothelium in response to wounds performed *in vitro* has many of the characteristics of such regeneration *in vivo*. By studying the migration of regenerating endothelial cells into intimal wounds produced *in vitro*, we have obtained new evidence in favour of the extracellular matrix as the short-term determinant of endothelial cell orientation. Moreover, although this apparent effect of the matrix can be modified temporarily by suitable wounding procedures, axial orientation cues persist even in the absence of blood flow.

Small pieces of rat aorta, pinned with glass needles to a Sylgard-coated culture dish and incubated in Medium 199 (supplemented with 30% heat-inactivated calf serum), maintain an intact endothelium, as determined by scanning and transmission electron microscopy, for as long as 10 days in culture (R.W.J., S. K. Anderson and J. D. Sheridan, in preparation). The endothelial cells respond to *in vitro* intimal wounds by migration and mitosis near the wound edge. A striking result is that the orientation of the regenerating cells depends on the type of wound produced.

With mechanical denudation it is theoretically possible to make four different wounds defined by the orientation of the wound edge and the direction of the mechanical stress used to make the wound. The edge of the wound can be oriented either circumferentially, as occurs in most experimental *in vivo* wounding procedures, or axially. The wound can be created by applying mechanical stress in the same direction as the edge or perpendicular to the edge. Thus, the following wounds are defined: (1) circumferential wounds made axially (the most common *in vivo* type³; see Fig. 1a); (2) circumferential wounds made circumferentially (not previously described; see Fig. 1b); (3) axial wounds made axially (related to the 'narrow scratch' wound recently reported⁸; see Fig. 1c); and (4) axial wounds made circumferentially (not previously described; see Fig. 1d). We have made all four types of wound in our aorta cultures and have monitored the endothelial regeneration for several days thereafter.

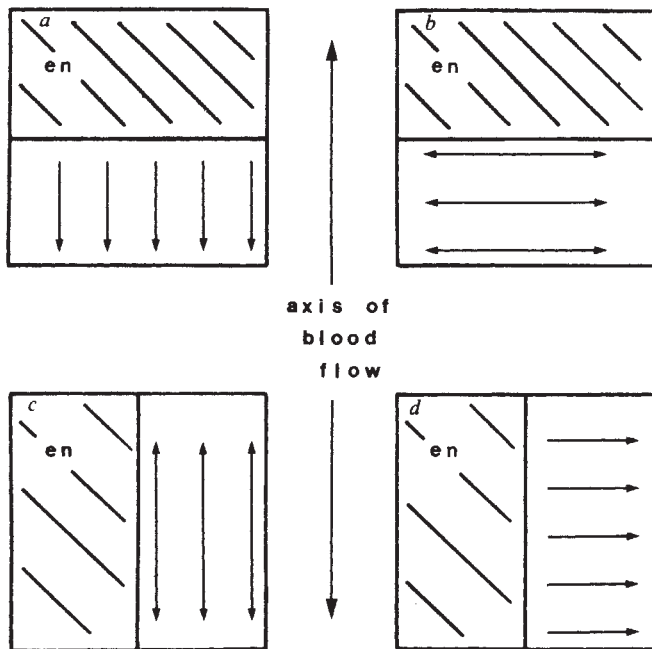
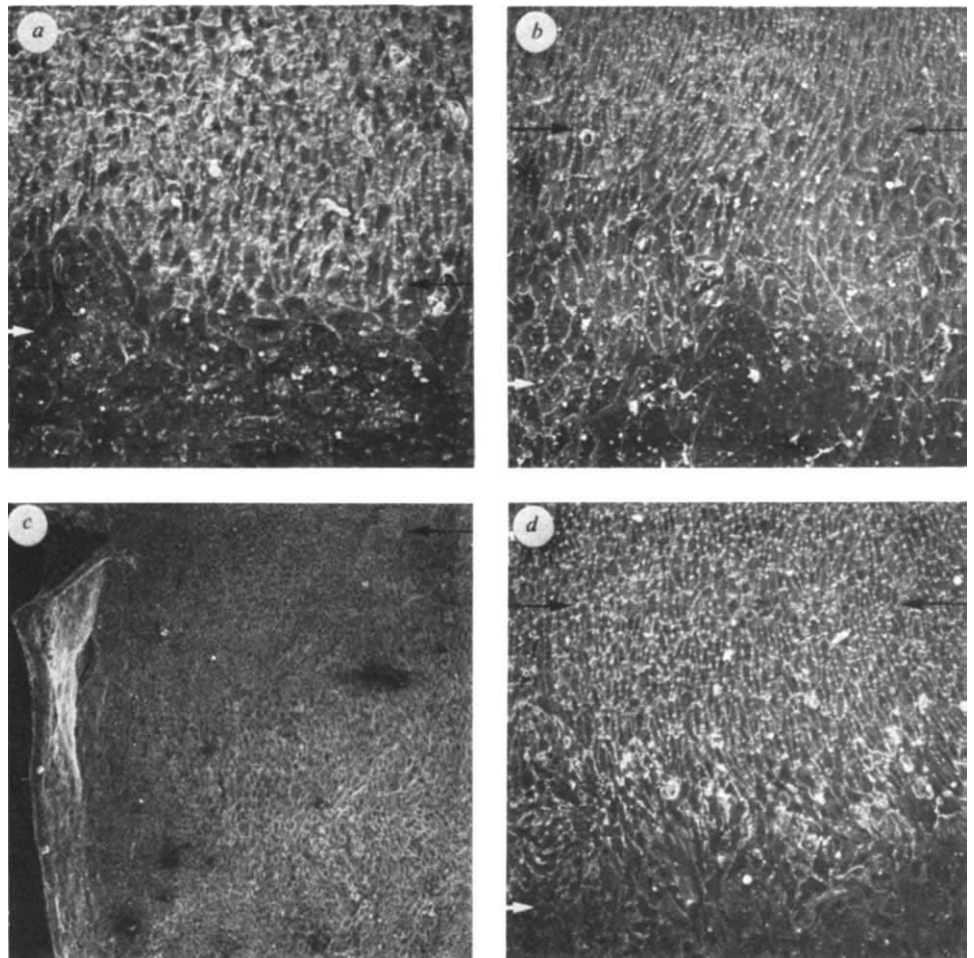


Fig. 1 Diagram of wounds made on cultured explants: *a*, circumferential wound edge made by wounding axially; *b*, circumferential wound edge made by wounding circumferentially; *c*, axial wound edge made by wounding axially; *d*, axial wound edge made by wounding circumferentially. Wounds were made with a rubber policeman at the 4th day *in vitro*. Unmarked arrows indicate the movement of the rubber policeman during wounding. Hatched area (en) indicates the endothelium remaining after wounding.

Figure 2*a-c* shows the results of regeneration from a circumferential wound made axially. After only 24 h of recovery (Fig. 2*a*) the endothelial cells have become elongated in an axial direction and have begun to migrate on to the denuded surface. These processes are even more apparent at 48 h after wounding (Fig. 2*b*). By 3 or 4 days, depending on the amount of surface removed originally, the endothelial cells completely resurface the explant (Fig. 2*c*). Figure 2*d* shows a circumferential wound made circumferentially. The preparation was fixed 48 h after wounding. It is apparent that orientation of the endothelial cells on the resurfaced area is almost exclusively axial. However, at the migrating front there is a greater degree of endothelial flattening and a more random endothelial orientation than when the wound is made axially.

When axial wounds are made, the axial and circumferential methods of wounding produce quite different results. If the axial wound is made axially there is little if any reorientation by 24 h after wounding (Fig. 3*a*). Any migration that has taken place has done so with a considerable axial component. By 72 h (Fig. 3*b*) the cells at the wound edge have maintained an axial or polygonal orientation and there has been only limited circumferential migration into the wound. Once again, any migration past the wound edge appears to have a strong axial orientation, suggesting that it is axial spill-over from the irregular wound edge. The resurfacing by the migrating cells has been inefficient and gaps remain exposing bare internal elastic lamina (IEL) in the re-established area. The failure of the endothelial cells to migrate into the wound does not result from some generalized damage of the IEL because, as shown (Fig. 3*b*), there is extensive axial growth from an accessory artery around which the surface was denuded during the wounding procedure. Although axial regeneration from this artery has been dramatic, as

Fig. 2 Scanning electron micrographs of explant surfaces wounded as in Fig. 1*a, b*. The explants were stained with silver nitrate and fixed in 2.5% glutaraldehyde in phosphate-buffered saline (PBS) for at least 4 h. This was followed by a post-fixation in 2% aqueous OsO_4 for 1 h. Fixed material was then rinsed several times in PBS, dehydrated in ethanol and critical point dried in CO_2 from amyl acetate. Preparations were sputter-coated with gold and viewed with an Hitachi S-450 scanning electron microscope. Black arrows indicate the approximate edge of the wound, estimated by the location of notches cut in the explant (see *c*). Rubber policeman wounds were begun along a line between these notches. Open arrows mark the position of the migrating wound front at the time of fixation (approximate, for irregular fronts). *a*, Circumferential wound edge made by wounding axially, explant fixed 24 h after wounding ($\times 133$). *b*, Wound edge as in *a*, explant fixed 48 h after wounding ($\times 90$). *c*, Wound edge as in *a*, explant fixed 72 h after wounding. Actual notch is visible in the upper left ($\times 34$). *d*, Circumferential wound edge made by wounding circumferentially, explant fixed 48 h after wounding ($\times 90$). In all cases, the vertical axis of the figures parallels the original blood flow axis for the vessels. The circumferential wound edge made by circumferential wounding was produced on explants from two separate rats. All the rest have been done on explants from at least three separate rats.



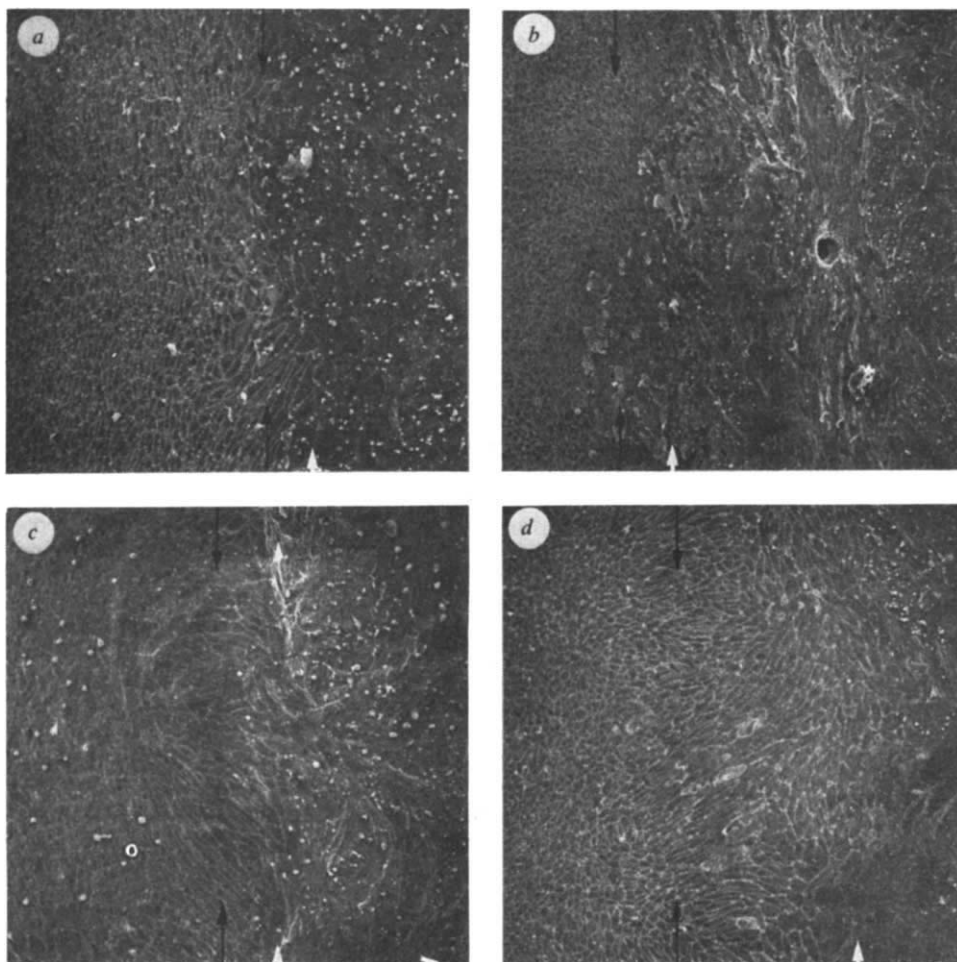


Fig. 3 Scanning electron micrographs of explant surfaces wounded as in Fig. 1c, d. The explants were prepared for scanning electron microscopy in the same way as for Fig. 2, and the black arrows once again indicate the placement of the original wound edge, while the open arrows give an estimate of the extent of the endothelial migration before fixation. a, Axial wound edge made by wounding axially, explant fixed 24 h after wounding ($\times 98$). b, Wound edge as in a, explant fixed 72 h after wounding ($\times 52$). c, Axial wound edge made by wounding circumferentially, explant fixed 24 h after wounding ($\times 82$). d, Wound edge as in c, explant fixed 48 h after wounding ($\times 55$). Again, in all cases, the vertical axis of the figures parallels the original blood flow axis for the vessels.

typically seen in *in vivo* wounds, circumferential migration from the same artery has not occurred.

For an axial wound made circumferentially, endothelial cells initially elongate and migrate circumferentially (Fig. 3c). By 48 h (Fig. 3d), however, two types of orientation are visible. Endothelial cells in the area of the original wound edge are oriented circumferentially, but at the edge of the regenerating endothelial sheet the cells are oriented axially. Between 48 and 72 h no further circumferential migration takes place, and consequently, part of the original wound area remains denuded. In contrast to the results for circumferential wound edges, we have observed that the extent of circumferential orientation in these wounds is influenced by the intensity of the wound. Light wounds produce much less circumferential orientation than do heavy ones.

It seems likely that the information affecting the orientation and migration of the endothelial cells on our explants must come from either the cells next to the migrating cells or some structure or cells below the endothelium. The former theory is supported by the results from circumferential wound edges, which demonstrate only orientation parallel to the orientation of the adjacent endothelium. In contrast, on explants with axial wound edges made circumferentially, the endothelial cells are clearly able to orient, and then reorient, in each case perpendicular to the orientation of the cells which would be presumed to be the source of orientation information. We feel, therefore, that the observations are more consistent with the source being beneath the endothelial cells, and present the following working hypothesis for the involvement of subendothelial information in the orientation and migration of regenerating endothelial cells on aortic explants.

When part of the endothelium is removed, cells along the wound edge elongate before migrating perpendicular to that edge. For circumferential wound edges made axially, axial

information is left intact, the elongation is pronounced and migration proceeds in a similar manner to that described for *in vivo* wound recovery². This seems to be a result of a combination of the tendency of migratory cells to invade a newly bared area (demonstrated for monolayer cultures without underlying matrix⁹) and contact guidance from axial cues⁴. In the case of circumferential wound edges made circumferentially, cells migrate in a near-normal manner but the more random orientation of cells at the migrating front suggests the partial loss of axial cues.

In the cases of wound edges which parallel the axial information, our observations support the idea that axial information not only directs endothelial migration, but may actually restrict it. For axial wound edges made axially, we believe that it is the persistent axial information which is sufficient to inhibit the elongation and migration perpendicular to the wound edge. In contrast, for axial wound edges made circumferentially, we propose that partial disruption of the axial information allows elongation and migration in a manner which seems to be due to a summation of factors similar to that for a circumferential wound made circumferentially, that is, compromised matrix information, and an heightened effect of the intrinsic tendency for cells to migrate into a bare area. Between 24 and 48 h in culture, the migrating cells of the circumferentially made axial wound edge reorient, once again aligning with the original axis of blood flow. This may indicate a regeneration of subendothelial information, or simply a change in the ability of the migrating endothelial cells to read the remaining axial information.

Other investigators have already demonstrated changes of endothelial orientation *in vivo* which must in some way involve blood flow forces^{10,11}. Our use of cultured explants has made it possible to eliminate blood flow from the factors responsible for the changes described here. However, two results from the present study seem to be important with regard to the relation-

ship between blood flow and subendothelial cues in the ultimate regulation of the orientation of endothelial regeneration *in vivo*. The first of these is the relative velocity of the reorientation with respect to these two variables. Changes induced by blood flow manipulation seem to require at least 3 days *in vivo*^{10,11} and 48 h for monolayers of endothelial cells¹². Reorientation in our experiments occurs in as few as 24 h and two such reorientations can take place in 48 h. Second, our observation of the inability of the cells to maintain a migration in the circumferential direction, here in the absence of blood flow, supports data from *in vivo* experiments showing that endothelial cells migrate effectively against blood flow but not circumferentially³. These two points strongly suggest that there are some aspects of endothelial regeneration which are not produced by the shear forces of blood flow; that is, the structure of subendothelial information may be modified by blood flow (see ref. 4), but it is the underlying matrix which carries the imperative of orientation for migrating endothelial cells.

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Are there purinergic receptors on parotid acinar cells?

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ATP and other purine nucleotides and nucleosides have potent regulatory (neurotransmitter-like) actions which have been attributed to interaction with a specific plasma membrane receptor¹. To date the receptor mechanisms underlying purinergic activation have been poorly characterized. One problem has been the variability of the evoked effects in different tissues. Burnstock^{2,3} proposed that much of the variability could be explained if the effects were mediated by two separate receptors, termed P₁ and P₂, with different specificities for agonists and antagonists. Receptor mechanisms have been extensively investigated in the parotid gland⁴. I now report that in that gland, ATP evokes a marked increase in membrane conductance, radioactive Rb efflux and amylase secretion. The effects of ATP are similar to those evoked by acetylcholine (ACh) and α -adrenergic agonists but are still present when cholinergic and adrenergic blocking agents are used. The latency and reversal potential of the ATP-evoked effects are comparable with those of the autonomic agonists. The ATP receptor on parotid acinar cells is of the P₂ type^{2,3}, since the order of potency of the nucleotide series was ATP > ADP > AMP, adenosine had no effect, and the response could be blocked by quinidine but not by theophylline.

Experiments were carried out on 249 isolated parotid glands from 152 adult male mice. All glands were superfused with oxygenated physiological saline solutions at 37 °C. The acinar cell response to the various agonists was investigated using three experimental techniques: (1) single glass microelectrodes were used to record transmembrane potential and input resistance from surface acinar cells⁵; (2) the rate of Rb efflux was monitored by measuring the efflux of ⁸⁶Rb from segments of prelabelled glands^{6,7}; and (3) amylase secretion was measured continuously by an on-line fluorometric method⁸. The agonists ATP, ADP, AMP, adenosine, ACh and adrenaline (Sigma) were applied by inclusion in the superfusion media, which had a final pH of 7.4. ATP, ACh and adrenaline were also applied ionophoretically⁹ from an extracellular micropipette.

The mean resting potential of the acinar cells was -62.2 ± 0.62 mV ($\pm$ s.e.m., $n = 74$) and the mean input resistance 4.6 ± 0.39 M Ω ($n = 19$), in close agreement with previously published values^{10,11}. Electrophysiological responses to ionophoretically applied ATP were observed in 69 cells from 18 glands. When the effects on the membrane of ATP and the autonomic agonists ACh and adrenaline were compared in 47 of these cells, they were all found to evoke a marked decrease in input resistance associated with an initial depolarization. This initial potential change was followed by a delayed hyperpolarization during which the input resistance returned to prestimulus levels (Fig. 1a). Such biphasic membrane responses have been previously reported in this tissue following cholinergic α -adrenergic and nervous stimulation^{9,10}.

In the remaining experiments the effects of ATP were investigated in the presence of combined autonomic blockade by atropine (10^{-5} M), phentolamine (10^{-5} M) and propranolol (5×10^{-6} M). The autonomic blockers had no effect on the resting membrane potential and input resistance, which were -66.3 ± 0.67 mV ($n = 94$) and 5.06 ± 0.31 M Ω ($n = 43$) respectively. It was necessary to demonstrate the persistence of the ATP effects in the presence of autonomic blockade because the peptide hormone substance P has been shown to evoke indirect effects in this tissue by release of endogenous neurotransmitter (ACh).¹¹ The initial potential change of the ATP response shows the same dependence on transmembrane potential gradient as has been reported for the cholinergic and α -adrenergic responses in this tissue. Figure 1b shows the ATP effects evoked in a single cell at different levels of membrane potential. In Fig. 1c the amplitude of the ATP effects is plotted as a function of the membrane potential. The reversal potential of the ATP effect was measured in this manner on five separate occasions, having a mean of -59.6 ± 4.1 mV (s.d.). This reversal potential is not significantly different from the values of -58.3 ± 9.0 and -61.9 ± 8.4 mV ($\pm$ s.d.) previously reported for ACh and adrenaline in mouse parotid gland¹⁰. The latency of the effects of ionophoretically applied ATP, in the presence and absence of the autonomic blockers, was 471 ± 33 ms ($\pm$ s.e.m., $n = 9$) and 489 ± 58 ms ($\pm$ s.e.m., $n = 15$) respectively, which is comparable with values reported previously for ACh and adrenaline in this tissue¹⁰. The identical latency in the presence and absence of the autonomic blockers excludes the possibility of any early indirect effects^{11,12}.

Superfusion of ATP in 55 cells from 37 glands in the presence of the combined autonomic blockers evoked a marked decrease in input resistance associated with a monophasic hyperpolarization similar to that reported following superfusion of cholinergic and α -adrenergic agonists¹³. Figure 2 shows the dose-response curve for the ATP-evoked hyperpolarizations at effective ATP concentrations of 10^{-5} – 10^{-3} M. The true dose-response curve is thought to lie to the left of the experimental one because (1) all investigations of the effects of exogenously applied ATP are complicated by hydrolysis or tissue uptake of the nucleotide²; (2) it has been reported that the α -adrenergic blocker phentolamine, at the concentration used routinely in this study to eliminate the possibility of indirect effects, will interact with the purinergic receptor¹⁴; and (3) ATP-evoked secretion in mast cells produces a similar dose-response

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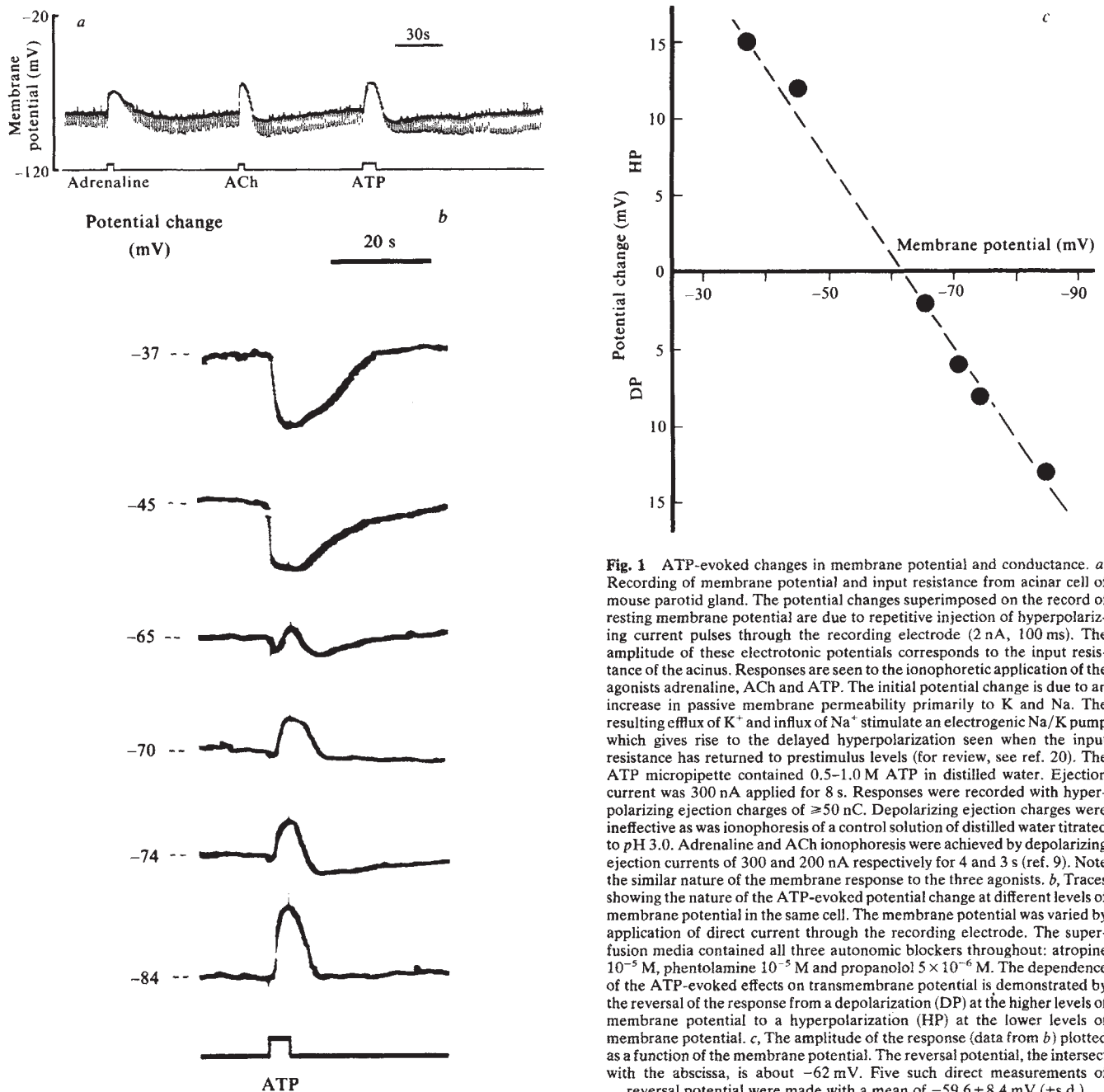


Fig. 1 ATP-evoked changes in membrane potential and conductance. *a*, Recording of membrane potential and input resistance from acinar cell of mouse parotid gland. The potential changes superimposed on the record of resting membrane potential are due to repetitive injection of hyperpolarizing current pulses through the recording electrode (2 nA, 100 ms). The amplitude of these electrotonic potentials corresponds to the input resistance of the acinus. Responses are seen to the ionophoretic application of the agonists adrenaline, ACh and ATP. The initial potential change is due to an increase in passive membrane permeability primarily to K and Na. The resulting efflux of K⁺ and influx of Na⁺ stimulate an electrogenic Na/K pump which gives rise to the delayed hyperpolarization seen when the input resistance has returned to prestimulus levels (for review, see ref. 20). The ATP micropipette contained 0.5–1.0 M ATP in distilled water. Ejection current was 300 nA applied for 8 s. Responses were recorded with hyperpolarizing ejection charges of ≥ 50 nC. Depolarizing ejection charges were ineffective as was ionophoresis of a control solution of distilled water titrated to pH 3.0. Adrenaline and ACh ionophoresis were achieved by depolarizing ejection currents of 300 and 200 nA respectively for 4 and 3 s (ref. 9). Note the similar nature of the membrane response to the three agonists. *b*, Traces showing the nature of the ATP-evoked potential change at different levels of membrane potential in the same cell. The membrane potential was varied by application of direct current through the recording electrode. The superfusion media contained all three autonomic blockers throughout: atropine 10^{-5} M, phentolamine 10^{-5} M and propranolol 5×10^{-6} M. The dependence of the ATP-evoked effects on transmembrane potential is demonstrated by the reversal of the response from a depolarization (DP) at the higher levels of membrane potential to a hyperpolarization (HP) at the lower levels of membrane potential. *c*, The amplitude of the response (data from *b*) plotted as a function of the membrane potential. The reversal potential, the intersect with the abscissa, is about -62 mV. Five such direct measurements of reversal potential were made with a mean of -59.6 ± 8.4 mV ($\pm$ s.d.).

curve^{15,16}, although in this case the true ATP agonist is the free form, the tetrabasic acid (ATP⁴⁻), which constitutes only a small fraction of the total ATP in solution ([ATP]). The [ATP⁴⁻] in the solutions used to obtain the curve in Fig. 2 (Ca 2.5 mM; Mg 1.37 mM) can be estimated as 2×10^{-7} – 2×10^{-5} M respectively¹⁵.

In parotid gland, as in other tissues, ⁸⁶Rb has been used as an indicator of potassium movement^{6,7}, and it is known that cholinergic and α -adrenergic agonists evoke a marked efflux of K (Rb) from acinar cells. Figure 3a demonstrates that when ATP is included in the superfusion media, even in the presence of the autonomic blockers, it mimics the effects of the autonomic agonists in its ability to evoke Rb efflux from the parotid tissue. Burnstock^{2,3} describes two types of receptor—the P₁ and P₂ purinergic receptors—which mediate the effects of exogenous and endogenous nucleotides and which are identified by their different specificities for the nucleotide/nucleoside series and their susceptibility to blockade by different agonists. The nucleotide series in evoking Rb efflux from this tissue is ATP > ADP > AMP (Fig. 3a). Adenosine had no effect even at 10^{-2} M (12 glands, 3 experiments), and failed to evoke any electro-

physiological effects in any of nine cells from four glands when included in the superfusion media at 10^{-2} M. Histamine (10^{-4} M) (8 glands, 2 experiments) also failed to evoke any increase in Rb efflux, eliminating the possibility that the ATP effects were secondary to release of endogenous histamine.

Figure 3b shows that when 10^{-3} M quinidine was included in the superfusion media, ATP failed to evoke any significant increase in Rb efflux (36 glands, 9 experiments); subsequent application of ACh (10^{-6} M), still in the presence of quinidine, evokes the characteristic increase in Rb efflux (20 glands, 5 experiments). In the presence of theophylline (10^{-3} M; Fig. 3b) the ATP effect persists, unaffected (16 glands, 4 experiments). The same specificity was seen in electrophysiological experiments. ATP failed to evoke any effect in three cells (two glands) in the presence of 10^{-3} M quinidine. However, the purinergic effects were readily evoked in the presence of theophylline (10^{-3} M), by both superfusion (six cells, three glands) and ionophoresis (two cells, one gland) of the agonist. Quinidine blockade, especially at the concentration used, is not specific and more prolonged exposure to quinidine (35 min) abolished the effects of α -adrenergic stimulation in this tissue, as in others¹⁷.

The persistence of the ACh effect at this time (Fig. 3b) and later would indicate that the non-specific interaction occurs at the receptor level and is not due to a generalized blockade of the effector mechanism.

In addition to the stimulus-permeability effects, cholinergic and α -adrenergic receptor activation are associated with amylase secretion, which is characterized by its dependence on extracellular calcium¹⁸. β -adrenergic stimulation, which is not associated with any of the conductance changes described above¹⁰, is a potent stimulator of enzyme secretion, but by a mechanism independent of extracellular calcium and attributed to interaction with the adenylate cyclase-cyclic AMP system¹⁹. ATP evokes a marked increase in amylase secretion (28 glands, 7 experiments) which exhibits the same dependence on extracellular calcium (20 glands, 5 experiments) as the other stimulus-permeability-coupled receptors (Fig. 4). Adenosine (10^{-2} M) had no effect on amylase secretion (8 glands, 2 experiments). The effects of the antagonists on amylase secretion could not be tested as quinidine itself interacted with the fluorometric assay.

Whereas a peptide (substance P) receptor was previously reported in rat acinar cells, but appeared absent from mouse acinar cells, in this study ATP effects were readily observed in the mouse parotid gland but could not be evoked in the rat. Superfusion of ATP (10^{-3} M) failed to evoke electrophysiological effects (6 cells, 2 glands), Rb efflux (4 glands, 2 experiments) or amylase secretion (4 glands, 2 experiments).

This study of mouse parotid gland reveals for the first time that ATP can mimic the effects of cholinergic or α -adrenergic stimulation in terms of increased membrane conductance, ^{86}Rb efflux and calcium-dependent enzyme secretion. The persistence of the ATP-evoked effects in the presence of a combination of autonomic blockers and their short latency (equivalent to autonomic activation) suggest that they are mediated by interaction of the nucleotide with a specific surface membrane receptor. The order of potency of the nucleotide series (ATP > ADP > AMP) and the susceptibility to blockade by quinidine but not theophylline indicate that a purinergic receptor of the P_2 type is involved^{2,3}. This type of purinoreceptor predominates in the gastrointestinal system². The identical nature of the membrane responses and the common reversal potential shared by the purinergic, cholinergic and α -adrenergic agonists suggest that all three effects, although mediated by separate receptors, are the consequence of the activation of the same effector

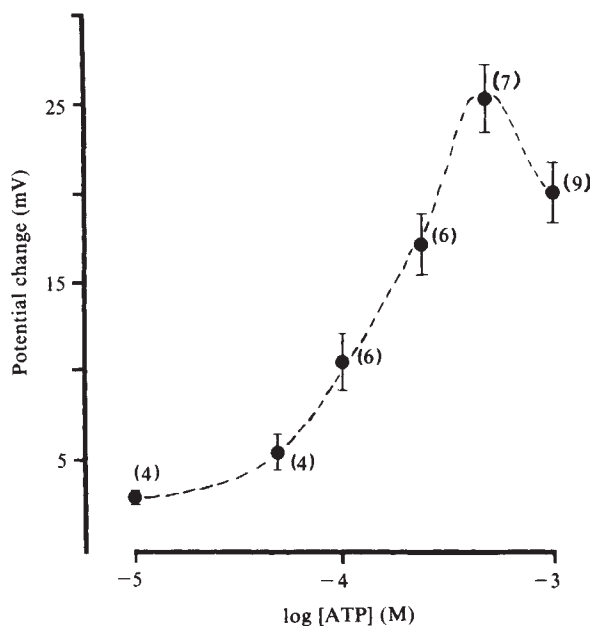


Fig. 2 Dose-response curve for hyperpolarization evoked in parotid acinar cells by superfusion with ATP (10^{-5} – 10^{-3} M). All experiments were carried out in the presence of the combined autonomic blockers. Figures in parentheses are the number of individual determinations. Plot is of means $\pm$ s.e.

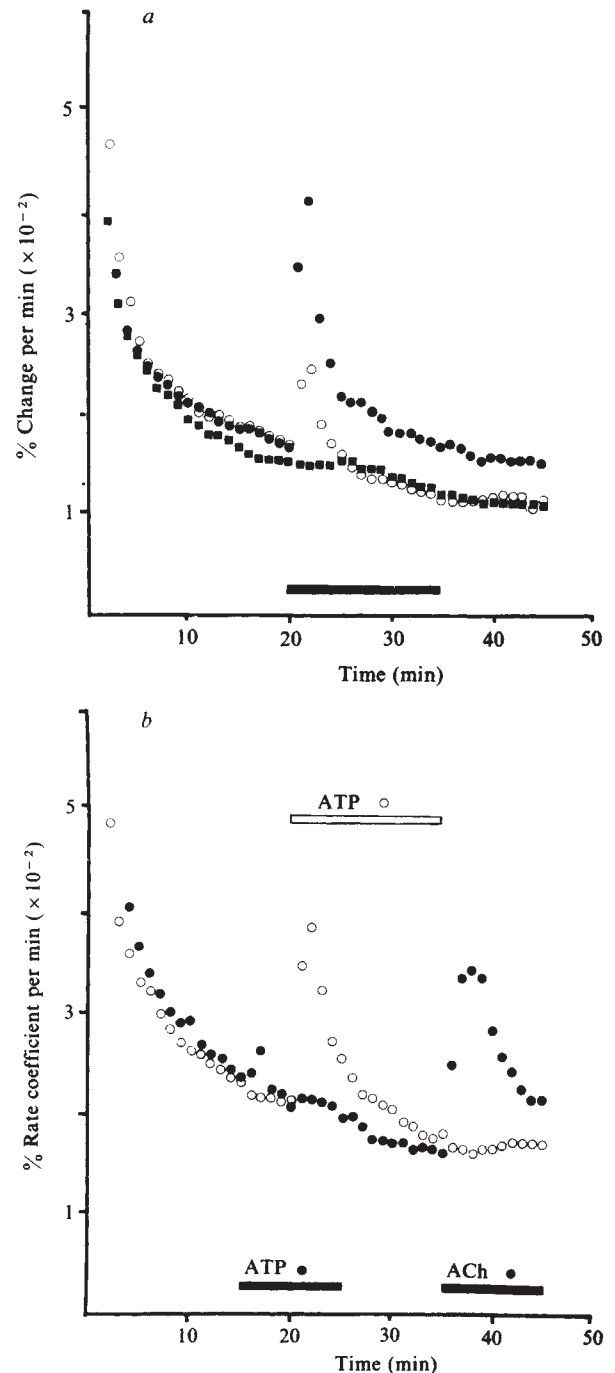


Fig. 3 Rate of ^{86}Rb efflux from segments of mouse parotid gland. In each experiment, segments of four glands were loaded with radioactive Rb for 30 min in 1 ml of physiological salt solution containing $3\text{--}5\text{ }\mu\text{Ci } ^{86}\text{Rb}$. After loading the segments were transferred to a flow chamber (volume 1 ml; flow rate 2 ml min^{-1}). The effluent was collected at 1 min intervals and subsequently analysed in a scintillation counter for ^{86}Rb content. The radioactivity remaining in the tissue at the end of the experiment was determined and the concentrations in the minute samples converted to rate coefficient⁶ by computer. a, Effects of purine nucleotides on the rate coefficient of ^{86}Rb efflux (given as % change per min). The agonists were applied by inclusion in the superfusion media at 20–35 min, as indicated by the bar in the figure. The autonomic blockers were present throughout the experiments. Each point is the mean of a series of experiments. ●, ATP (10^{-3} M; $n=5$); ○, ADP (10^{-3} M; $n=3$); ■, AMP (10^{-2} M; $n=3$). (s.e. during application of the agonists was 5–15%.) The order of potency of the nucleotide series was ATP > ADP > AMP. Neither adenosine (10^{-2} M) nor histamine (10^{-4} M) had any effect. b, Effect of ATP (10^{-3} M) on ^{86}Rb efflux in the presence of quinidine (●; 10^{-3} M; $n=5$) and theophylline (○; 10^{-3} M; $n=4$). Quinidine blocks the effect of ATP applied at 15–25 min. ACh (10^{-6} M) applied in the same experiments at 35 min still evokes the characteristic increase in ^{86}Rb efflux. When applied at 20–35 min in the presence of theophylline, ATP evokes the characteristic increase in ^{86}Rb efflux. The affinities demonstrated for the agonists (ATP > ADP > AMP) and antagonists indicate that the effects are mediated by a P_2 purinergic receptor^{2,3}.

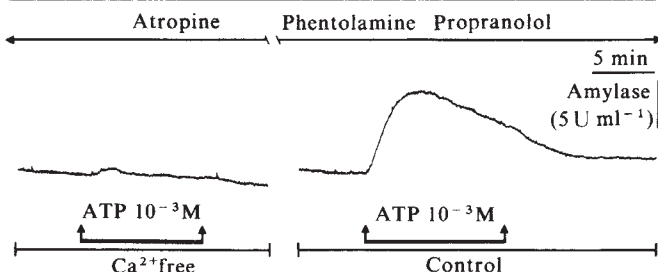


Fig. 4 Effects of ATP on amylase release from segments of mouse parotid gland. The amylase content of the effluent from the tissue chamber (volume 1 ml; flow rate 1 ml min⁻¹) was continually assayed by an automated on-line fluorometric method⁸. The figure which is two sections of a continuous trace, shows the effects of ATP in the presence and absence of extracellular calcium from the superfusing solution. The calcium-free solution contained 10⁻⁴ M EGTA. The autonomic blockers were present throughout the experiment. The ATP-evoked increase in amylase secretion is seen to depend on extracellular calcium.

mechanism. These stimulus-permeability-coupled receptors give rise to an increase in membrane permeability primarily to K and Na by a mechanism which probably utilizes Ca as a common intracellular messenger²⁰.

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Neural mechanisms for serial order in a stereotyped behaviour sequence

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A single, transient sensory stimulus can evoke a complex behavioural response in which several behavioural units occur in a serially ordered manner. For stereotyped behavioural sequences, two alternative neural mechanisms have been proposed to account for the serial ordering of behavioural units: chain reflexes¹, which depend on peripheral feedback, and central pattern generators², which attribute the sequence to interactions among central neural elements. Although long thought to be mutually exclusive, both alternatives were recently shown to contribute to simple crayfish escape responses^{3–5}. We now demonstrate that the integration of these simple, serially ordered escape responses into the compound escape sequence of the crayfish is mediated by a third mechanism for serial order, namely the parallel activation by the same sensory stimulus of two separate behavioural units with different intrinsic reaction times.

Crayfish can escape from predators by swimming backward using a series of rapid abdominal flexions and extensions (tailflips). When escape is initiated at short latency by a sudden tactile stimulus to the abdomen, an initial stereotyped tailflip is mediated by a pair of giant axons, while all subsequent tailflips (swimming proper) are produced by a non-giant central pattern generator^{3,4}. The giant axons have been termed 'command neurones'⁶ because, when stimulated directly, they produce an entire, coordinated tailflip. However, we recently showed that only the flexion phase of the first tailflip is produced by central action of the giants; abdominal re-extension is a chain reflex that is triggered by sensory feedback from the flexion⁵.

Proper behavioural integration requires that the non-giant central pattern generator for swimming be turned on at the right time following the first giant-mediated tailflip. How is this achieved? We considered three possibilities. First, the giant axons might trigger the central pattern generator (CPG) via a delayed, central pathway. Second, the CPG, like the re-extension reflex, might be triggered by feedback from the first tailflip. Third, the tactile, initiating stimulus might trigger both the giant axon system and the CPG in parallel. Evidence for the third alternative was obtained by stimulating directly and selectively the giant axons of unrestrained crayfish via implanted electrodes^{4,5} and comparing the resulting escape responses with those initiated by natural stimuli.

Twenty crayfish, *Procambarus clarkii* (of both sexes; 3–7 cm long), were kept individually in well-aerated, 40-l aquariums. Each animal was made to respond only once per 10 min. Approximately half of the responses were produced by lightly tapping the abdomen; the other half were produced by a direct shock to the giant axons. Responses were monitored visually and via chronically implanted recording electrodes.

The result was an almost complete dichotomy in the incidence of multiple tailflips produced by natural and electrical stimuli. Following taps, ~82% of the escape responses consisted of an initial, giant-mediated tailflip followed by one or more non-giant (CPG-mediated) tailflips. But when the giant axons were stimulated directly, only a single tailflip ensued; CPG-mediated swimming was almost completely absent, occurring in <1% of all trials (Table 1). These results mean that activity in the giant axons is insufficient to trigger the CPG either via a central pathway or via feedback. Instead, excitation arising from the tactile stimulus is necessary for CPG activation.

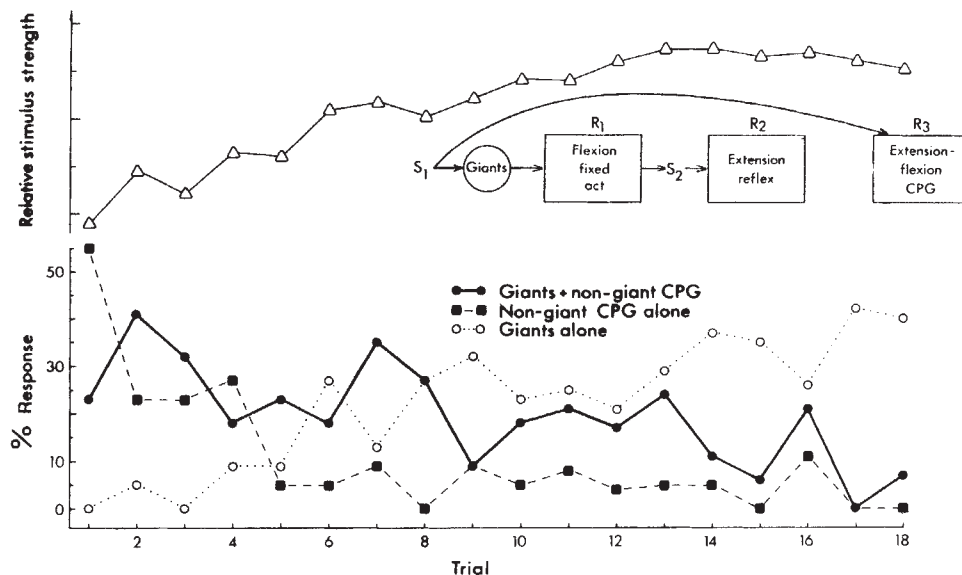
Is the tactile stimulus sufficient to trigger the CPG, or must it be coupled with sub-threshold excitation produced by the giant axons? In the preceding experiment, only 4% of the responses were mediated by the CPG alone, which may mean that the tactile stimulus is rarely sufficient to activate the CPG, or that the stimuli used were typically supra-threshold for both the giant axons and the CPG.

We attempted to activate the systems separately by reducing the stimulus strength so that it was just at escape threshold (50% responding), and by testing at 1-min intervals so that thresholds rose during a testing session (presumably because of habituation). Four animals were tested in this way. The results (Fig. 1) show that the tactile stimulus is frequently sufficient to trigger the CPG. Indeed, in rested animals the CPG threshold is lower than the giant axon's threshold. In the first trial, 71% of the escape responses were mediated exclusively by the CPG and 100% included the CPG (Fig. 1). (Repetitive stimulation caused the threshold for the CPG to rise relative to the LG threshold,

Table 1 Different response modes produced by central and peripheral stimulation

Type of stimulus	Type of response		
	Giants alone	Giants + non-giant	Non-giants alone
Shock to giant axons (n = 445)	99.3%	0.7%	—
Touch to abdomen (n = 453)	13.9%	82.1%	4.0%

Fig. 1 The block diagram illustrates the relationships between the units of behaviour that make up the crayfish escape response. The initiating stimulus activates R_1 and R_3 in parallel, but R_3 occurs only after a delay. R_2 is a chain reflex that depends on sensory feedback from R_1 . The graph shows differences in initial thresholds and habituation rates of giant-mediated responses ($R_1 + R_2$) and non-giant(CPG)-mediated responses (R_3). In the experiment shown, each of four animals was tested at 1-min intervals on five successive days. Thus each trial represents the average obtained from ~20 stimuli, given as per cent responsiveness. The stimulus strength (top) was increased over trials in order to maintain a total escape response rate close to 50%. Total response rates varied between 77% (trial 1) and 45% (trial 12). By keeping the stimulus close to threshold we were able to demonstrate the separate response pathways. Stimulus strength is indicated in relative units (peak initial amplitude of the output of a mechano-electrical transducer which was coupled to the mechanical stimulator). The three main results are: (1) the systems can be evoked independently; (2) the CPG threshold is initially the lowest; and (3) the CPG habituates more rapidly.



thus solo activity of the CPG quickly waned, with solo activity of the giant system eventually predominating.)

Given that the same stimulus can activate both behaviours in parallel, how is the serial order of the compound behaviour achieved? Previous results can now be re-interpreted to show that an intrinsic difference in response latency is an important mechanism for the sequential ordering of the behavioural units. The giant axon pathway has obviously evolved to produce responses of very short latency: the pathway is short, its neurones are large, and all known synapses except those of receptors and motoneurons appear to be electrical^{7,8}, resulting⁴ in a modal response latency of <7 ms. In contrast, the minimum latency for activation of the non-giant CPG is ~50 ms and the modal latency is >150 ms (see ref. 4). (The neural basis for the long latency of the CPG has not yet been investigated.) The latency values reported by Wine and Krasne⁴ have been confirmed in our experiments, which are based on a much larger sample size. For 180 giant-mediated tailflips, the mean latency was 6.3 ± 0.2 ms. For 63 exclusively non-giant responses, the mean latency was 240 ± 21 ms. The mean duration of the giant-mediated tailflip (flexion and re-extension), occurring in isolation, was⁵ 190 ± 21 ms. Thus, it is clear that the typical latency to the beginning of CPG-mediated tailflips is long enough to allow the first two behavioural units to occur in the interim.

These results extend our understanding of how serial order is achieved in stereotyped behaviour. A previous hypothesis¹ to explain how a transient sensory stimulus could lead to ordered behavioural responses proposed that sequencing occurred because each reflex caused feedback appropriate to trigger the next reflex. Convincing examples of chain reflexes are rare^{5,9} and so far have been restricted to episodic behaviours. A second hypothesis is that order is imposed by a central pattern generator, envisaged as one or more neural oscillators. This hypothesis has been confirmed for every rhythmic behaviour whose production has been analysed carefully². Both these mechanisms are used in simple crayfish escape responses. In addition, a third mechanism—parallel activation by the same sensory stimulus of two separate neural subsystems with different reaction times—is responsible for the integration of a chain reflex and a central pattern generator into a seemingly unitary complex behavioural act.

What is the adaptive significance of the parallel processing strategy? We know that the giant axon system ignores the laterality of the stimulus and produces a bilaterally symmetrical response, whereas non-giant responses are laterally asymmetric and can steer the animal away from threat^{4,10}. Therefore, the

parallel strategy may be required because the stimulus contains information that is used by the second system but not by the first.

It seems likely that reaction-time mechanisms for serial order occur widely. For example, the timing of two distinct components of the hours-long, hormonally-induced eclosion behaviour of a moth appears to rely on a reaction-time mechanism¹¹. Thus this general type of mechanism seems capable of ordering behavioural units into complex sequences of action on time scales which vary from milliseconds to hours.

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FMRFamide neuropeptides simultaneously increase and decrease K^+ currents in an identified neurone

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Phe-Met-Arg-Phe-NH₂, or FMRFamide, discovered in ganglia of the clam *Macrocallista nimbosa*¹, has potent cardio-stimulatory activity in some molluscan species, but is cardio-inhibitory in others, and produces contractions of certain non-cardiac muscles². A peptide with similar properties is present in the ganglia of the snail *Helix*³, and is thought to differ from the *Macrocallista* peptide in having one or more additional amino acids combined with the terminal free amino nitrogen. An

homologous opiate peptide, Tyr-Gly-Gly-Phe-Met-Arg-Phe (or YGGFMRF, using single letter abbreviations⁴), was identified in adrenal medullary granules and the striatum⁵. YGGFMRFamide has chromatographic properties more similar, though not identical, to the *Helix* peptide than FMRFamide itself (D. A. Price, M. J. Greenberg and G.A.C., unpublished). FMRFamide has potent and complex effects on *Helix* neurones. One neurone in each cerebral ganglion, the C₁ or giant serotonin neurone (GSN), is hyperpolarized by FMRFamide at the resting membrane level, but depolarized at less negative potentials⁶. I show here that YGGFMRFamide has similar effects to FMRFamide and examine further the ionic mechanisms underlying the two responses of the GSN. The results indicate that the hyperpolarizing, outward current response results from an increase in conductance to K⁺ whereas the depolarizing response, which was recorded as a reduction in net outward current, is due to the suppression of a Ca²⁺ activated K⁺ current.

In most experiments the neurone was voltage-clamped using a Dagan 8100 single electrode system with K-acetate or KCl electrodes of ~1 MΩ (see ref. 7). However, responses were also recorded with a two-electrode system which incorporated either the amplifier of a Tektronics 502A oscilloscope or an RS-7611 C-Moss amplifier, in the feedback circuit. Physiological solution (NaCl 80 mM, KCl 5 mM, CaCl₂ 7 mM, MgCl₂ 5 mM, Tris buffer 5 mM, final pH 7.8) was allowed to flow continuously over the isolated cerebral ganglia preparation pinned to the base of a 0.7-ml chamber. The peptides were applied locally to the GSN either by iontophoresis (4 mM in distilled water) or by pressure ejection from a pipette (50–100 μM concentrations of each peptide).

Each peptide hyperpolarized the GSN at the resting potential. The size of the hyperpolarizing response was increased when the external K⁺ concentration was reduced, but remained unchanged when the external Cl⁻ concentration was reduced. Under voltage-clamp, the reversal potential of the peptide-induced outward current was increased by -35 to -38 mV when the external K⁺ was changed from 5 to 1 mM (see, for example, Fig. 1b). This shift in reversal potential is close to the theoretical value of -41 mV and indicates that K⁺ is primarily, if not exclusively, involved in the hyperpolarizing response to each peptide, although the reversal potential of the response seems to be more positive for this neurone than that recorded for K⁺ responses in some other molluscan neurones (see, for example, ref. 8). The outward current response was depressed with tetraethylammonium bromide (TEA) at 30 mM, but was not completely blocked even with 100 mM TEA. It was also reduced by cooling to 4 °C. The other action of the peptides was observed at potentials less negative than the resting level. This apparent inward current response increased in size as the membrane was depolarized. Frequently a combination of both outward and 'inward' current responses was observed (Fig. 2c, d). TEA at 30 mM greatly reduced the response due to the increase in K⁺ conductance^{9,10} and revealed an enlarged, or in some cases a previously undetected, response which was inward in sign (Fig. 2a). The size of the apparent inward current response was not reduced when the external NaCl was replaced with sucrose or glucosamine hydrochloride, suggesting that neither Na⁺ nor Cl⁻ is directly involved in the response. An involvement of Ca²⁺ did, however, seem likely, as CoCl₂ at ≥1.5 mM reversibly abolished this response (see Fig. 2b and ref. 11). On the other hand, when BaCl₂ was substituted for most of the CaCl₂ (6 mM BaCl₂ 1 mM CaCl₂), the response was also abolished. This suggested that the response was not simply due to an influx of Ca²⁺, but that it could result from a suppression of a K⁺ current, as Ba²⁺ can carry the Ca²⁺ current in snail neurones¹² but blocks K⁺ currents¹³. A role of K⁺ was also suggested by the results of 2 out of more than 60 experiments made with FMRFamide, in which only the inward current response was recorded. In these two experiments, the size of the response at a holding potential of -30 mV was greatly increased on reducing the external K⁺ concentration from 5 to 1 mM.

There is some variability in the sensitivity of the delayed K⁺ and the Ca²⁺ activated K⁺ currents to different concentrations of TEA in molluscan neurones. In some neurones, both currents are equally affected whereas in others the Ca²⁺-activated K⁺ current seems to be less susceptible^{9,10}, which could explain the preferential reduction in the outward current response with

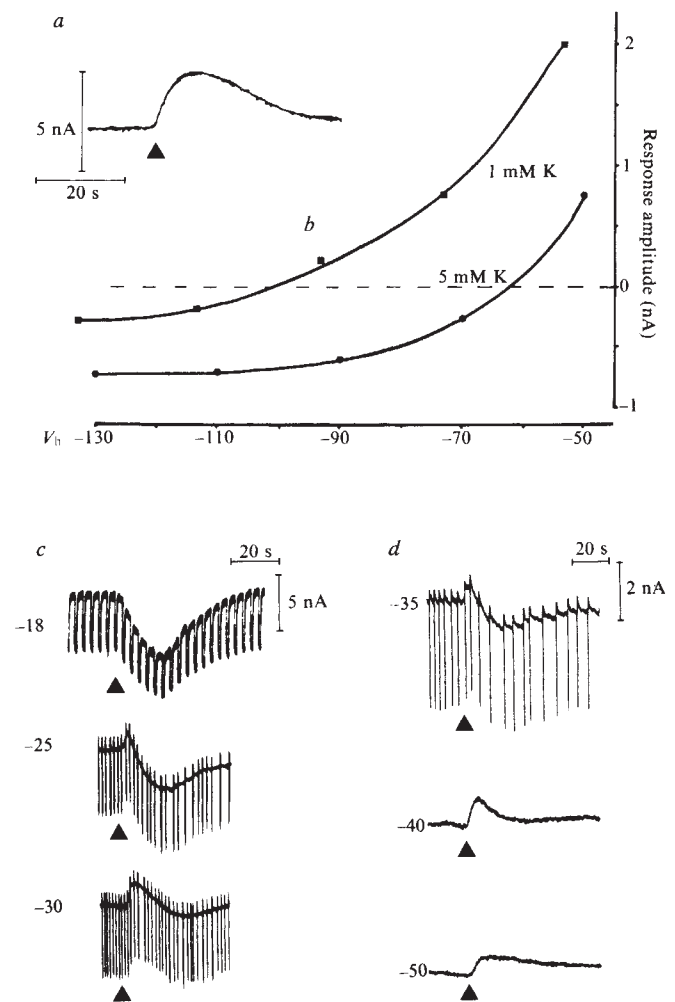
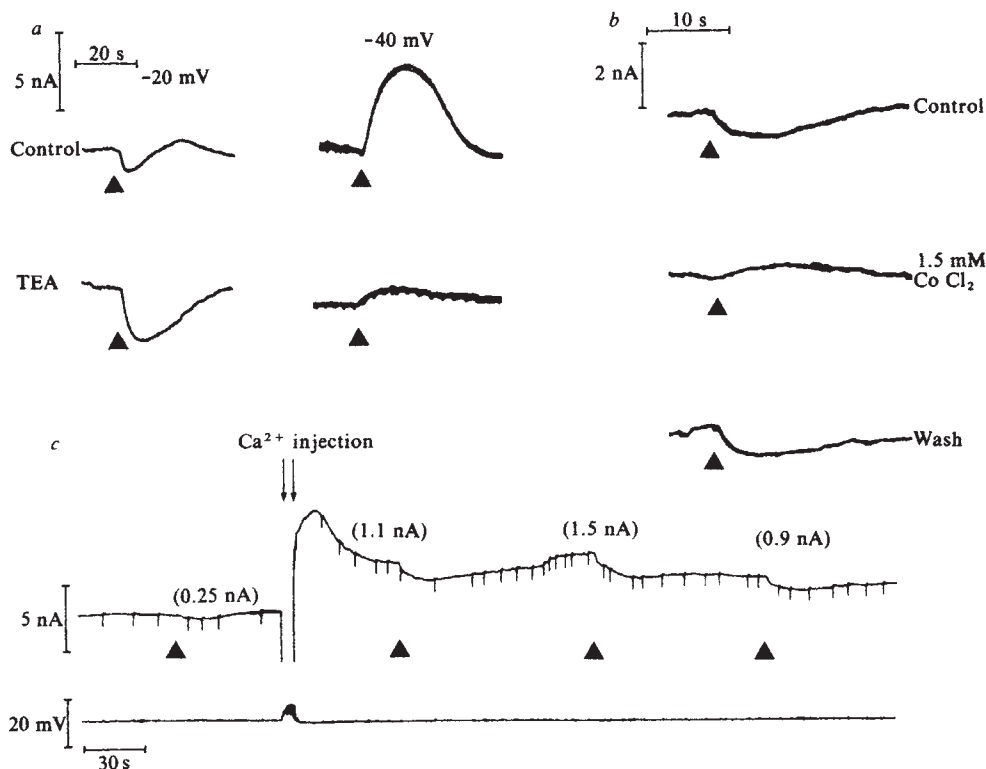


Fig. 1 a, An example of the outward current response recorded when FMRFamide was locally applied to the GSN at a holding potential of -50 mV. b, Influence of holding potential and extracellular K⁺ concentration on the size and sign of this response observed in another experiment. The reversal potential in normal medium (5 mM K) was ~-62 mV. When the extracellular K⁺ concentration was reduced to 1 mM, the reversal potential increased to ~-100 mV. The nonlinear relationship between response and membrane potential is similar to that previously shown for a carbachol-evoked K⁺ response in *Aplysia* by Ginsborg and Kado⁸. c, FMRFamide responses recorded at depolarized holding potentials. At -30 mV, the outward current response was shortened to give a small response, inward in sign. At -25 mV, the 'inward current' response was greatly increased and the outward current response was reduced. At -18 mV, the response seems to consist of an 'inward current' response alone. During this recording, hyperpolarizing command voltage steps were repeatedly applied across the membrane and the resulting changes in current recorded. During the action of the peptide, there appears to be a reduction in current passage. These recordings should be compared with the recording in a which was obtained from the same neurone. (The downward deflection on the current records at -25 and -30 mV holding potentials are unclamped spikes originating in the axon, at some distance from the site of recording.) d, Similar responses of another neurone to locally applied YGGFMRFamide. At -50 mV, there seems to be only an outward current response. At -35 mV, the response was predominantly inward in sign. The outward current response was shortened by the onset of the inward current response in the recording made at the holding potential of -40 mV. Note that in the traces shown in c and d, and also in Fig. 2, responses are recorded on top of steady outward currents. Such background currents varied to some extent from cell to cell, but with a resting potential of -50 mV they were of the order of 1.5 nA at -40 mV, 5 nA at -30 mV and 20 nA at -20 mV.

Fig. 2 *a*, Influence of 30 mM TEA on the responses of the GSN to FMRFamide applied at holding potentials of -20 and -40 mV. At -20 mV, the control response was a composite of currents which were inward and outward in sign, whereas at -40 mV the response seems to be purely outward. Exposure to TEA for 8 min, reduced the outward response at -40 mV and unmasked a response which was inward in sign at -20 mV. *b*, Influence of 1.5 mM CoCl_2 solution on the response. At the holding potential of -20 mV, the response was inward in sign. Exposure to the CoCl_2 solution reduced the background outward current observed at -20 mV by about 10 nA. It also abolished the inward current response and unmasked a small outward current response. The effect of the CoCl_2 was reversed by washing. *c*, Effect of locally applied YGGFMRFamide on the increased K^+ current evoked by intracellular injection of Ca^{2+} . Upper trace is membrane current and the lower trace membrane voltage. The neurone was voltage-clamped at -45 mV and the peptide applied locally to the neurone. Before injecting Ca^{2+} , there were only small inward current responses to the peptide. (The outward current response had been depressed with 30 mM TEA.) Injection of Ca^{2+} (40 nA for ~ 6 s) resulted in an increase in the background outward current, that is, the Ca^{2+} -dependent K^+ current. Application of the peptide at this time resulted in a greatly increased inward current response, that is, a suppression of the increased outward K^+ current. The amplitude of each peptide response is indicated above the upper trace.



30 mM TEA on the GSN (Fig. 2*a*). Of the two compounds, the YGGFMRFamide was the more potent in inducing the response which was inward in sign but less potent with respect to the outward current response.

These data suggested that the apparent inward current response could result from a suppression of a Ca^{2+} -activated K^+ current. Support for this view was obtained by testing the effect of the peptides on the increased K^+ current evoked by intracellular injection of Ca^{2+} in the presence or absence of TEA. In these experiments, Ca^{2+} were injected iontophoretically using a WPI iontophoretic programmer. An electrode containing 0.5 M CaCl_2 was inserted in the neurone and a positive current of 40 nA passed for 5–15 s with respect to another micropipette placed in the medium. The cell was voltage-clamped during the injection and the membrane potential therefore remained unchanged, or was only shifted by about 5 mV during the injection period. The Ca^{2+} -evoked outward current response was reversed in sign at about -65 mV in normal medium and was reversed at more negative potentials in medium with reduced KCl, as previously observed in other neurones¹⁴, establishing that the Ca^{2+} -evoked current was due to K^+ . An example of an experiment showing the effect of the heptapeptide amide on the increased outward current evoked by Ca^{2+} injection is seen in Fig. 2*c*, in which the neurone was clamped at -45 mV and a small inward current recorded before injecting Ca^{2+} . Following Ca^{2+} injection, there was an increase in outward K^+ current and the inward current effect of the peptide was potentiated as much as six times. As the increased outward K^+ current declined, the effect of the peptide was reduced. It was also observed that the peptide reduced the inward current evoked by Ca^{2+} injection when the neurone was voltage-clamped at -90 and -100 mV.

From these data it is concluded that both FMRFamide and YGGFMRFamide increase one K^+ current, which has properties of the delayed K^+ current^{9,10}, but suppress another, probably the Ca^{2+} -dependent K^+ current described by Meech^{14,15}. Interestingly, the polypeptide apamin, a neurotoxin of bee venom, has been reported to block the Ca^{2+} -dependent

K^+ channels in hepatocytes and intestinal smooth muscle¹⁶. Muscarinic agonists also suppress a K^+ current, the M-current in frog sympathetic neurones, although this current is not Ca^{2+} -dependent¹⁷. The significance of the dual actions of FMRFamide and YGGFMRFamide is not known, but it is possible that if the two types of action occurred on different axonal processes of the GSN there could be a suppression of transmission via the routes on which increased conductance to K^+ occur, but a potentiation of transmission on the routes which respond with a reduction in the Ca^{2+} -dependent K^+ conductance. The GSN has a large number of axonal processes, many of which run in parallel for long distances along some nerve trunks¹⁸. It is thought that FMRFamide and related peptides may act as hormones in molluscs², and immunoreactivity to FMRFamide has been detected histochemically in gastropod neurones¹⁹.

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Selective blockage of voltage-dependent K⁺ channels by a novel scorpion toxin

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Blocking agents of high selectivity are crucial in defining both physiologically and biochemically the molecular components that control membrane excitability. To obtain such probes for voltage-dependent ion channels, we have examined the venom of several American scorpions for the presence of polypeptide neurotoxins having the required properties. We report here that using voltage-clamped giant axons of the squid *Loligo vulgaris* we have identified in the venom of the scorpion *Centruroides noxius* Hoffmann a polypeptide (fraction II-11) that specifically depresses the peak permeability of K⁺ channels without affecting their voltage-dependent open-close kinetics. The venom also contains a polypeptide toxin (fraction II-10) that specifically depresses Na⁺ peak permeability with only minor effects on the activation-inactivation kinetics. Furthermore, the physiological effects of the whole venom on the squid giant axon can be assigned quantitatively to the combined action of the two polypeptides.

The venom of *C. noxius* Hoffmann was purified as described previously¹. Using gel permeation and ion-exchange chromatography, we isolated seven major fractions containing polypeptides toxic to mice (fraction II-8 to II-14). Of these only the minor fractions II-10 and II-11 (5 and 2%, respectively, of the total venom protein) exerted significant effects on the electrophysiological properties of the squid giant axon in the concentration range tested. Fractions II-10 and II-11 each contain only one polypeptide, but it is possible that the former also contains very small amounts of a contaminant. The N-terminal sequences of toxins II-10 and II-11 were shown to be respectively Lys-Glu-Gly² and Thr-Ile-Ile (L.D.P., unpublished observation).

Figure 1a-c shows the reversible effects of toxin II-11 on the K⁺ current, I_K , of a tetrodotoxin (TTX)-poisoned squid giant axon voltage-clamped as described elsewhere^{3,4}. On step depolarization, toxin II-11 (1.4 μ M) applied externally reversibly depressed the K⁺ permeability, g_K , without affecting its voltage dependence (Fig. 1d). Potassium permeability was depressed in a dose-dependent manner (Fig. 1e), suggesting interaction of the toxin with specific receptor site(s) associated with the K⁺ channel. This view is further supported by the binding and dissociation kinetics (Fig. 1f) at two different toxin concentrations. The rate of toxin association increased with increasing toxin concentration whereas the time constant of dissociation remained constant⁵. In contrast to the effects on K⁺ channels, toxin II-11 had no significant effect on Na⁺ peak permeability when tested separately on three axons internally perfused with Cs ions to block K⁺ currents. In addition, neither Na⁺ nor K⁺ permeability were affected by intracellular application of toxin II-11 (1.4 μ M).

Only one other polypeptide (fraction II-10) of the *Centruroides noxius* venom exerted a sizeable effect on ion channels of the squid giant axon. Figure 2 shows that toxin II-10 interacted with receptor site(s) at the Na⁺ channel, thereby reducing peak Na⁺ conductivity in a dose-dependent manner (Fig. 2d), indepen-

dently of the voltage (Fig. 2c). The activation kinetics were influenced only marginally (Fig. 2a, b) and the maintained current levels were affected only at higher concentrations (data not shown). However, both fast and slow inactivations were unmodified and neither the holding potential nor the external Na⁺ concentration changed toxin binding⁶⁻¹¹. Together with its reversible action, toxin II-10 thus seems to have advantages over other scorpion toxins as a probe for Na⁺ channels.

In experiments using whole venom, the actions of the venom were fully reversible and the same as those ascribed to toxins II-10 and II-11, and as observed previously for a different scorpion venom¹², the size but not the kinetics of gating currents was modified by the venom.

Various effects on voltage-dependent ion channels have been reported for scorpion venoms and purified toxins⁶⁻¹⁵. Na⁺ channel blockage is rather complex and varies with the scorpion

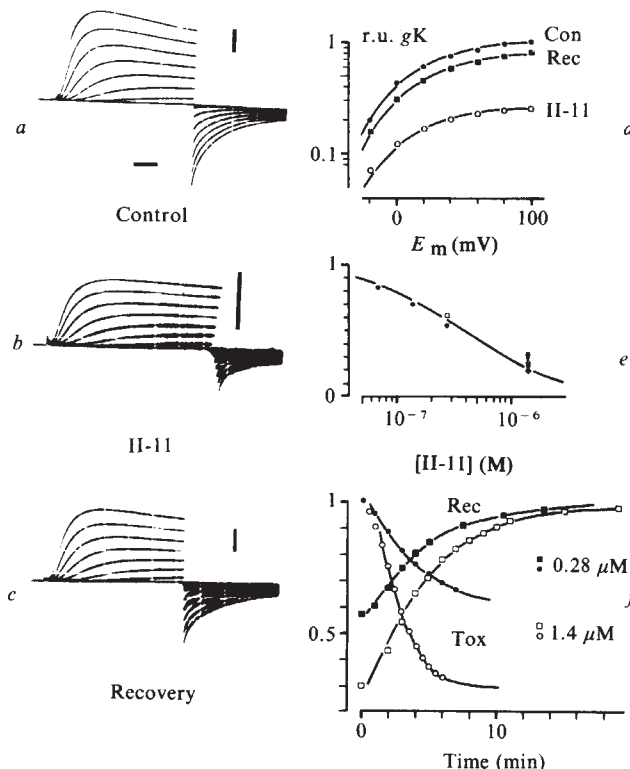


Fig. 1 Action of toxin II-11 on K⁺ channels. a-c, I_K recorded at various test potentials (-20, 0, 20, 40, 60, 80 and 100 mV) before, during and after application of 1.4 μ M of the toxin. Traces in b were obtained 10 min after exposure to II-11 and traces in c, 22 min after the onset of recovery. Scale bars, 1 mA and 2 ms. $E_h = -70$ mV, $E_c = -90$ mV for 80 ms. Successive stimuli were separated by 3 s intervals. Positive feedback and analog compensation for leakage and capacitive currents were used. In (mM): KF (317); sucrose (307); K phosphate (45), pH 7.2. Out (mM): NaCl (435); KCl (10); CaCl₂ (10); MgCl₂ (40); Tris-HCl (20), pH 8.0; TTX (3×10^{-7} M). $T = 5 \pm 1$ °C. d, Dose-response curve of toxin II-11. Ordinate, relative g_K at $E_m = 100$ mV. The solid line is the result of a curve fit using $K_D = 390$ nM and the equation: $(1 + 10^{\log [TX] - \log K_D})^{-1} = g_K(TX)/g_K(C)$, where [TX] is toxin concentration, and $g_K(C)$ and $g_K(TX)$ the value of potassium conductance for the control and in the presence of toxin, respectively. K_D is the equilibrium dis-

sociation constant of the reaction $TX + R \xrightleftharpoons[k_2]{k_1} TX \cdot R$; where R is the concentration of receptor sites. f, Onset and offset of toxin binding measured at $E_m = +100$ mV. Ordinate, $g_K(t)$ normalized to the value at time zero. Solid lines are exponentials with time constants of 120 s (1.4 μ M) and 240 s (0.28 μ M) for the onset and 300 s for the offset. Tox, toxin. For clarity, complete recovery was assumed and records in c were normalized to 1 accordingly.

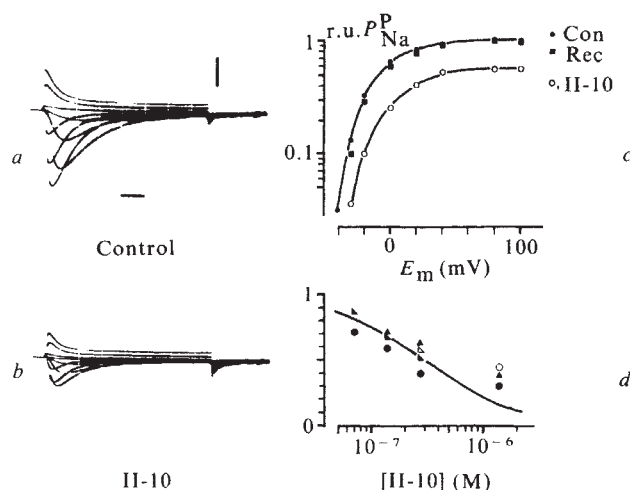


Fig. 2 Action of toxin II-10 on Na^+ channels. *a, b*, Na^+ currents recorded before and during application of 280 nM of toxin II-10. Traces in *b* were obtained after 14 min exposure to fraction II-10. Recovery was complete within 35 min (not shown). Scale bars, 1 mA and 2 ms. The axon was held at -70 mV (E_h) and pre-conditioned to -90 mV for 80 ms (E_c) before delivering test potentials (E_t): -40 , -30 , -20 , 0 , 20 , 40 , 60 , 80 and 100 mV. Successive stimuli were separated by 2 s. In (mM): CsF (267); NaF (50); sucrose (307); K-phosphate (45), pH 7.2. Out (mM): same as in Fig. 1 but without TTX. $T = 5^\circ\text{C}$. *c*, Relative P_{Na}^r , peak sodium permeability, of records *a* and *b* as derived using the Goldman-Hodgkin-Katz equation¹⁹; $E_{\text{Na}} = 58$ mV. The solid line drawn through the open circles is the same as that drawn to fit the control data but scaled in amplitude and shifted 10 mV along depolarizing voltages. *d*, Dose-response curve of toxin II-10. Different symbols denote different axons. Ordinate, relative P_{Na}^r at $E_m = 100$ mV. Abscissa, molar concentration of toxin.

species^{8,15}. K^+ channel blockage has only been described together with Na^+ channel blockage for whole Scorpion venom^{5,13} and a toxic fraction of another venom¹⁴. Thus the important finding of the present study is that toxin II-11 acts exclusively on K^+ channels without any significant effect on Na^+ channel characteristics. In addition, K^+ channel blockage is complete, independent of the applied voltage and fully reversible. These properties make toxin II-11 an excellent tool for physiological and biochemical studies of the K^+ channel of excitable cells.

Compared with TTX binding to Na^+ channels⁵ and snake toxin binding to the acetylcholine receptor^{16,17}, toxins II-11 and II-10 bind to their target sites on the squid axon with relatively low affinity ($K_D = 300$ nM and 390 nM, respectively) and with small association rate constants. This may be due to conformational rearrangements of the toxins required during attachment to the ion channels of the squid. More typical preys of the scorpion may have channels to which binding requires no such conformational adjustments and hence may have a larger association rate constant and lower K_D ¹⁸.

Despite these minor reservations, polypeptides II-11 and II-10 seem to have advantages over previously described probes for the study of voltage-dependent Na^+ and K^+ channels. They have simple mechanisms of action, are fully reversible and, most important, II-11 is the first polypeptide toxin found to affect the voltage-dependent K^+ channel of excitable membranes.

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Triphosphoinositide increases glycoprotein lateral mobility in erythrocyte membranes

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The finding¹ that the turnover of the anionic phospholipid, phosphatidylinositol (PI), increases with increased synaptic activity has long stimulated interest in the possible functional roles of this phospholipid and its phosphorylated products, phosphatidylinositol 4,5-bisphosphate (triphosphoinositide or TPI) and phosphatidyl-myoinositol 4-phosphate (diphosphoinositide or DPI)². TPI metabolism is altered by a variety of cellular stimuli², but no specific function of the lipid has been identified. However, in many instances (see, for example, refs 3-6) a correlation has been observed between changes in PI metabolism and membrane structure. We have recently correlated membrane macro-viscosity with the lateral mobility of membrane glycoproteins⁷, and have shown that 2,3-diphosphoglycerate (2,3-DPG), which is similar in structure to TPI, increases glycoprotein lateral mobility⁸. We now report that TPI also increases the lateral mobility of glycoproteins when added to erythrocyte membranes, and suggest that TPI acts similarly to other polyanions by disrupting the erythrocyte membrane skeleton.

The method of fluorescence redistribution after photobleaching (FRAP) was used to measure the lateral mobility of erythrocyte membrane glycoproteins labelled with dichlorotriazinylaminofluorescein (DTAF) as previously described⁸⁻¹⁰. We have recently shown that fluorescence photobleaching does not alter the lateral mobility of the band 3 glycoprotein in erythrocytes¹¹. The fluorescent label was primarily (65-75%) on band 3, the major anion channel of the erythrocyte, with the remainder on glycophorin and an unknown component⁸.

Erythrocytes were lysed (1:100) in hypotonic solutions (1 part phosphate-buffered saline (PBS, 140 mM NaCl, 10 mM Na_2HPO_4 , pH 7.4) and 2 parts 5 mM Na phosphate pH 7.4 with 1 mM EDTA) containing sonicated phospholipid suspensions of PI (Sigma) or ³²P-labelled DPI and TPI at different concentrations. ³²P-TPI and -DPI were prepared from rat brain or from erythrocyte membranes by the method of Schacht¹², or by preparative TLC. Lipid preparations were judged to be 90% of the designated phospholipid (see Fig. 1) by two different TLC systems using commercial standards of TPI and DPI (Sigma). Sonication was performed for 10-15 min at 24 °C under N_2 in a bath sonicator to produce a clear suspension. After lysis of the cells in the presence of the lipid, membranes were incubated for 10 min at 37 °C to allow lipid incorporation and binding to occur.

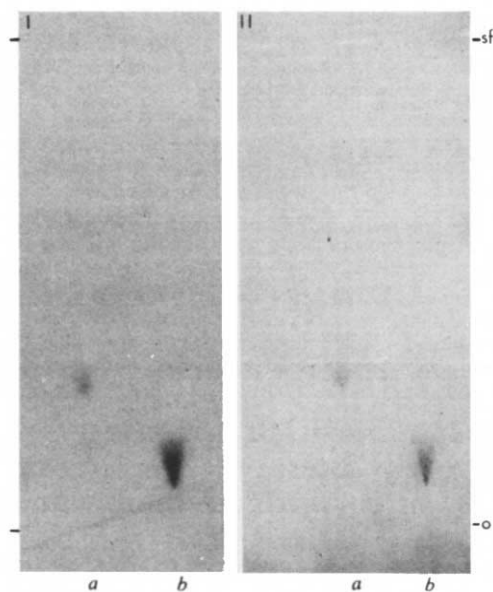


Fig. 1 TLC of ^{32}P -labelled DPI (a) and TPI (b) prepared by the method of Schacht¹². I is an autoradiogram and II is the material visualized by a modified Dittmer-Lester spray¹⁸. After purification, the column fractions were dried under N_2 and spotted on silica gel H (Sigma, without oxalate, 0.25 mm thick layer). Plates were developed with 1-propanol, 4 M ammonium hydroxide (2:1)¹⁹. Sigma standards of TPI and DPI had R_F values of 0.19 and 0.33 in this system.

FRAP measurements were performed on the membranes after incubation without washing. The presence of increasing amounts of TPI in the lysis medium increased the apparent diffusion coefficient of the integral protein-bound label by more than twofold (Fig. 2). The range of diffusion coefficients for the control sample without sonicated lipid was $2.1\text{--}9.5 \times 10^{-11} \text{ cm}^2 \text{ s}^{-1}$ whereas with 10^{-6} M TPI the range was $9.4\text{--}44 \times 10^{-11} \text{ cm}^2 \text{ s}^{-1}$. In a paired t -test the average values were significantly different ($P > 0.001$). Figure 2 clearly shows that TPI is much more effective than DPI in increasing glycoprotein lateral mobility.

The protein diffusion coefficient is unchanged when TPI is added to erythrocytes which are swollen but not lysed in a hypotonic solution (equal parts PBS and 5 mM PO_4 , pH 7.4, with 1 mM EDTA) ($4 \times 10^{-11} \text{ cm}^2 \text{ s}^{-1}$ compared with $4 \times 10^{-11} \text{ cm}^2 \text{ s}^{-1}$ for control in this experiment). This suggests that

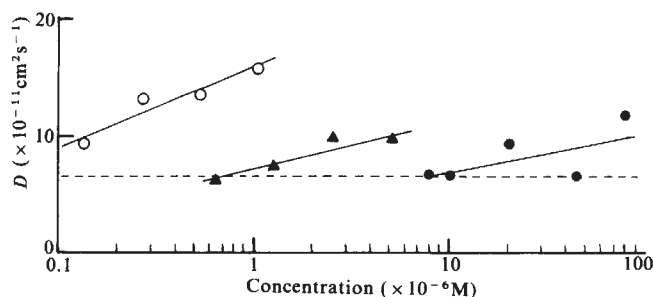


Fig. 2 The apparent lateral diffusion coefficients (D) of DTAF-labelled membranes are plotted against the concentration of TPI (○) and DPI (▲), prepared by the method of Schacht¹², and PI (●; Sigma). Each point is the average of four determinations. The control value ($n = 19$) is shown by the dotted line. After pretreatment with diisopropylfluorophosphate and labelling with DTAF, cells were lysed by diluting 1:100 in sonicated suspensions of the lipids in 46 mM NaCl, 5 mM Na_2HPO_4 and 1 mM EDTA (pH 7.4). Membranes were preincubated for 10 min at 37°C before the FRAP measurement.

the active agent must reach the interior membrane surface. The observed increases in protein diffusion could not have been caused by contaminants of the lipid preparations (ammonia, ammonium acetate and neomycin). Ammonia and ammonium acetate will readily cross membranes and thus would be expected also to affect unlysed membranes. Neomycin and other amines have been shown previously to reduce the lateral mobility of membrane glycoproteins⁸. In addition, TPI prepared by preparative TLC of erythrocyte membranes also caused an increase in the lateral mobility.

By analogy with previous results with 2,3-DPG, we suggest that TPI increases the lateral mobility by disrupting linkages in the erythrocyte membrane skeleton. Both TPI and 2,3-DPG have the same -5 valency, and a similar charge group structure (Fig. 3). Both are effective in increasing the lateral mobility of membrane proteins only when they have access to the cytoplasmic surface of the membrane, the surface of the extrinsic membrane matrix⁷. In many related studies, we and others have observed that 2,3-DPG and other polyanions disrupt the spectrin-actin and band 4.1 complex which comprises the erythrocyte membrane matrix¹³⁻¹⁵.

The major difference between the 2,3-DPG and TPI experiments is in the concentrations needed to produce the effects observed: 2,3-DPG will disrupt the erythrocyte membrane matrix and increase glycoprotein mobility when present in

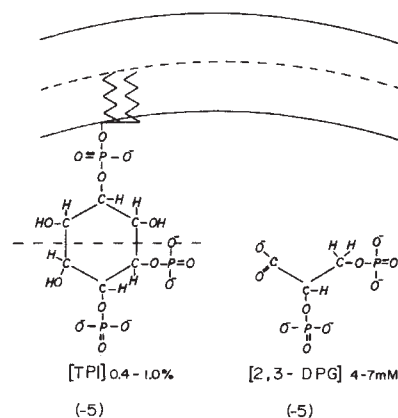


Fig. 3 The chemical structures of the lipid head group of TPI and 2,3-DPG are shown with their valency and normal *in vivo* concentration in erythrocytes. The concentration of TPI is expressed as a percentage of total erythrocyte lipid.

millimolar concentrations, whereas TPI is effective at the micromolar level. This difference could be explained if TPI is incorporated into the membrane bilayer, thus serving to increase its effective concentration at the membrane surface where the spectrin network is located. To assay for incorporation into cells we lysed the cells in the presence of ^{32}P -TPI. After incubation for 10 min at 37°C , the membranes were placed on a linear sucrose gradient (1–30% sucrose in 5 mM PO_4 , pH 7.4, with a 50% sucrose cushion) and centrifuged to equilibrium (30 min at 110,000g). The low ionic strength lysed the membranes, which would then float at a density of $\sim 1.15 \text{ g ml}^{-1}$. With both 4 and 40 μM TPI per ml in the incubation mixture, 10% of the TPI was associated with the membrane fractions (1% cells). It is not known whether all this lipid is integrated into the membrane bilayer but it is expected that a fraction of it will be.

This information allows us to calculate the total amount of cell-associated TPI. At a concentration of 10^{-6} M , there are $\sim 10^5$ TPI molecules per swollen red cell volume, whereas in the erythrocyte membrane there are normally 10^6 TPI molecules per membrane (0.5% of total phospholipid). After incubation, approximately the equivalent of 10 cell volumes of TPI is associated with the membranes, doubling the amount of TPI in

the membrane. If some of the TPI is complexed with glycoporphin^{16,17}, the added TPI may increase the amount of free TPI by more than twofold. The binding of TPI to the membrane bilayer through its hydrophobic tail will serve to increase its concentration at the membrane surface where the spectrin network is located; $1-2 \times 10^6$ molecules of TPI corresponds to an effective concentration of 4–8 mM in a 50 Å space on the internal surface of the membrane, which in turn is the concentration range where 2,3-DPG is effective.

Thus, TPI is a potent activator of glycoprotein lateral diffusion in erythrocyte membranes, and probably acts by disrupting linkages in the erythrocyte membrane skeleton. This effect may have important consequences for such vital red cell characteristics as cell shape and deformability. In a wider context, we suggest that TPI may have a similar effect on membrane skeletal complexes in other types of cells.

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Phosphorylation inhibits guanine nucleotide exchange on eukaryotic initiation factor 2

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There is extensive evidence that protein synthesis in reticulocyte lysates is inhibited during haem deficiency because of phosphorylation of initiation factor eIF-2 by a protein kinase (the haem-controlled repressor, HCR)¹⁻⁵. Other protein kinases, which are activated by low concentrations of double-stranded RNA, have also been identified in reticulocytes^{1,6} and in extracts from interferon-treated cells⁷⁻¹⁰, and these phosphorylate an identical site on eIF-2¹¹⁻¹². Despite the extensive documentation of such enzymes, however, the precise mechanism of inhibition has remained unclear, mainly because phosphorylation fails to affect many partial reactions of polypeptide chain initiation^{13,14}. We present here evidence that the exchange of GTP for GDP bound to eIF-2 is inhibited following phosphorylation of the factor. Such an effect may inactivate eIF-2 by impairing the ability to recycle between successive rounds of protein synthesis.

It has been proposed previously that the recycling of eIF-2 between successive initiation events, rather than the formation

of initiation complexes *per se*, is impaired by phosphorylation. Little evidence for this, other than indirect kinetic arguments¹⁵, has, however, been presented. We have been investigating the roles of the guanine nucleotides, GTP and GDP, in regulating eIF-2 activity and have previously suggested that an important step in the recycling of the factor is the need to exchange GTP for GDP^{16,17}. The former nucleotide is required for formation of pre-initiation complexes between eIF-2, initiator Met-tRNA_i, 40S ribosomal subunits and mRNA^{13,14,18} and is then hydrolysed to GDP and phosphate on or before joining of the 60S ribosomal subunit¹⁹. The eIF-2 is released from the ribosome at this stage and may remain associated with the GDP, for which it has a high affinity²⁰. GDP is a potent inhibitor of eIF-2 activity²¹ and would have to be removed very efficiently *in vivo* before the initiation factor could catalyse another round of protein synthesis. It is not known how this is achieved in the intact cell, but *in vitro* eIF-2 activity is enhanced by energy-generating systems such as phosphoenol pyruvate/pyruvate kinase¹⁷, phosphocreatine/creatine kinase²² and nucleoside diphosphate kinase²¹ (which rephosphorylate free GDP), or an enzyme which hydrolyses GDP to GMP and phosphate¹⁷. We have now developed model systems intended to reflect the process of guanine nucleotide exchange on eIF-2 *in vivo* and have investigated the effects on this process of phosphorylation of eIF-2 by HCR *in vitro*.

GDP displacement from eIF-2 was assayed by incubating eIF-2 with this nucleotide in a three stage experiment. The first stage consists of binding radiolabelled GDP to the initiation factor; binding is complete within 10–15 min at 30°C, as measured by retention of radioactivity on cellulose nitrate filters. In the second stage the [eIF-2·GDP] complex is incubated with or without HCR and ATP. Finally, the bound GDP is displaced by adding excess unlabelled GTP (with or without initiator Met-tRNA_i) or GDP and the kinetics of nucleotide exchange are measured. Figure 1 shows that phosphorylation of eIF-2 after incubation with ³H-GDP has no initial effect on the amount of radioactivity already bound to the factor. The [eIF-2·GDP] complexes are also stable on further incubation (Fig. 1b). However, when unlabelled GTP or GDP is added, the labelled nucleotide is displaced with kinetics which depend on the state of phosphorylation of the factor (Fig. 1a, c). As shown in Fig. 1a, the initiation factor exchanges bound ³H-GDP for non-radioactive GTP, in the presence of Met-tRNA_i, over a period of 15–25 min. After treatment with HCR the [eIF-2·GDP] complexes are much more stable and do not readily exchange with GTP. In the absence of Met-tRNA_i, the behaviour of the [eIF-2·³H-GDP] complexes is very similar (Table 1). Phosphorylation of eIF-2 with HCR also prolongs the half life of [eIF-2·³H-GDP] complexes during exchange with a sevenfold molar excess of unlabelled GDP (Fig. 1c, Table 1).

Experiments in which eIF-2 is incubated with HCR and [^γ-³²P]ATP and then analysed by SDS-gel electrophoresis

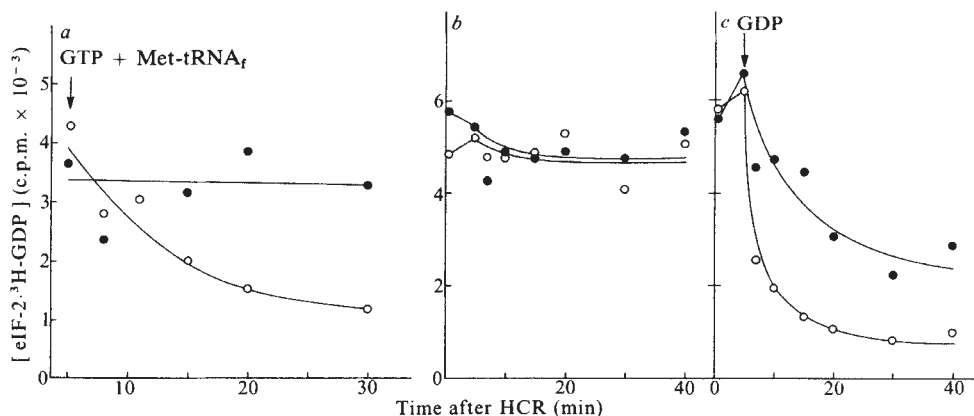
Table 1 Effects of HCR treatment on displacement of ³H-GDP from eIF-2

Expt	Additions at:		³ H-GDP bound at:		% ³ H-GDP remaining at 25 min
	10 min	15 min	10 min (c.p.m. × 10 ⁻³)	25 min	
1	—	GTP	4.24	1.62	38
	HCR	GTP	3.61	2.95	82
2	—	GTP	3.89	2.06	53
	HCR	GTP	3.59	3.78	105
3	—	GTP + Met-tRNA _i	4.31	2.00	46
	HCR	GTP + Met-tRNA _i	3.65	3.17	87
	—	GDP	2.69	0.80	30
	HCR	GDP	3.60	1.89	53
4	—	GDP	5.83	1.34	23
	HCR	GDP	5.60	4.48	80

The data summarize the results of several experiments carried out as described in Fig. 1 legend. Initiation factor eIF-2 was incubated for 10 min at 30°C with ³H-GDP in the presence of 0.2 mM ATP; HCR (8 U ml⁻¹) was then added where indicated for a further 5 min. At this time the bound ³H-GDP was displaced from eIF-2 by addition of: expt 1, 2 μM GTP (freshly prepared); expt 2, 50 μM GTP (free of contaminating GDP), with or without 12.3 nM unlabelled Met-tRNA_i; expts 3 and 4, 2 μM unlabelled GDP.

Fig. 1 Effects of HCR on displacement of ^3H -GDP from complexes with eIF-2. Initiation factor eIF-2 was prepared from a 15-l suspension culture of Ehrlich ascites tumour cells by suspending pelleted ribosomes in 14 ml of high salt buffer (0.25 M sucrose, 0.005 M MOPS pH 7.6, 0.5 M KCl, 0.005 M Mg-acetate, 0.001 M dithiothreitol (DTT), 0.1 mM EDTA) and re-centrifuging at 147,000g for 2 h. The supernatant (salt wash) was fractionated by ammonium sulphate precipitation between 30% and 70% saturation, dissolved in 8 ml of 0.02 M MOPS, 0.35 M KCl, 0.5 mM DTT, 0.1 mM EDTA, dialysed overnight against this buffer and then subjected to chromatography on phosphocellulose (Whatman P11; 16 $\times$ 42 mm column).

The column was washed with the above buffer, the eIF-2 was eluted with a 50-ml gradient of 0.35–0.8 M KCl in the above buffer and activity was detected by ternary complex formation (carried out in the conditions described in ref. 17). The fractions containing activity (eluting at 0.56–0.7 M KCl) were pooled, dialysed for 1 h against 0.05 M KCl in buffer, diluted with an equal volume of buffer without KCl and applied to a 3.5-ml column of DEAE-cellulose (Whatman DE-52). This column was washed with 0.09 M KCl in buffer, and eIF-2 was eluted with a 25-ml gradient of 0.09–0.4 M KCl in buffer and assayed as above. A peak containing 80% of the activity was pooled and precipitated by dialysis against 70% saturated ammonium sulphate in 0.02 M MOPS, pH 7.4. It was dissolved in 1 ml of buffer containing 0.09 M KCl and further dialysed against the latter for 1 h. The factor preparation, which was 30–50% pure, was stored in small aliquots in liquid nitrogen. The GDP displacement assay was carried out in three stages. (1) ^3H -GDP (6.7 $\mu\text{Ci ml}^{-1}$, 0.47 μM in *a*; 5 $\mu\text{Ci ml}^{-1}$, 0.35 μM in *b* and *c*) was incubated with eIF-2 (~12 μg of protein) in 150 or 200 μl for 10 min at 30 $^{\circ}\text{C}$ in the presence of 0.02 M HEPES pH 7.6, 0.1 M KCl, 0.002 M Mg-acetate, 0.5 mM DTT, 0.1 mM EDTA and 0.2 mM ATP. (2) The incubation was then continued for a further 5 min in the presence or absence of 8 U ml^{-1} of partially purified HCR (1 unit gives 75% inhibition of protein synthesis in a reticulocyte lysate after 60 min). (3) Additions were then made of 50 μM GTP plus 12.3 pmol ml^{-1} unlabelled Met-tRNA_i (*a*), nothing (*b*) or 2 μM unlabelled GDP (*c*). At the times shown, 20- μl samples were withdrawn, diluted in 1 ml of cold wash buffer (0.02 M HEPES pH 7.6, 0.1 M KCl, 0.002 M Mg-acetate) and immediately filtered on 0.45 μm cellulose ester filters (Millipore). The filters were washed with 3 $\times$ 3 ml of wash buffer, dried and the bound radioactivity determined. *a*, Kinetics of dissociation of [eIF-2- ^3H -GDP] complexes in the presence of GTP and Met-tRNA_i; *b*, [eIF-2- ^3H -GDP] complexes with no added unlabelled GDP; *c*, kinetics of dissociation of [eIF-2- ^3H -GDP] complexes in the presence of unlabelled GDP. $\circ$, Control eIF-2; $\bullet$, HCR-treated eIF-2.



show that the presence of bound GDP, GTP or Met-tRNA_i does not affect the extent of phosphorylation of the factor in the conditions of the assays described in Fig. 1 and Table 1 legends (data not shown). Furthermore, no net phosphate is lost from eIF-2 during the longer third stage of the incubations. The data presented in Table 2 show that inhibition of guanine nucleotide exchange requires not only HCR but also ATP, and the latter must be present in a hydrolysable form. When ATP is absent or is replaced by its non-hydrolysable analogue, β , γ -methylene adenosine triphosphate, GDP displacement by GTP is unaffected by HCR treatment. These results are consistent with a need for HCR-catalysed phosphorylation of eIF-2.

A potentially significant aspect of this work is that these results have been obtained with preparations of eIF-2 which are not completely homogeneous. SDS-gel electrophoresis indicates, in addition to the major eIF-2 bands, the presence of several polypeptide components with molecular weights from 47,000 to 97,000 (data not shown). Furthermore, similar experiments using highly purified (homogeneous) eIF-2 show slow GDP exchange kinetics whether or not the factor has been phosphorylated. These results suggest that nucleotide exchange may be catalysed by another component, analogous in its action to the polypeptide elongation factor EF-Ts^{23–25}. This component may be present in our partially purified eIF-2 but absent from the homogeneous factor, and the interaction between this component and eIF-2 could be sensitive to the state of phosphorylation of the latter. Several laboratories have reported the existence of one or more accessory proteins^{26–30} which stimulate eIF-2 activity when the initiation factor is assayed at low (physiological) levels in the presence of Mg²⁺, and which interact with the unmodified but not the phosphorylated form of eIF-2^{26,28–30}. Recently, a very similar factor has been characterized in the ribosome wash from Ehrlich cells and procedures have been developed for its separation from eIF-2 (L. R. V. Panniers and E.C.H., unpublished data). We are now therefore investigating the possibility that this factor acts by catalysing guanine nucleotide exchange on unmodified, but not phosphorylated, eIF-2.

During this work we have observed that not only the dissociation of GDP but also the binding of this ligand to our eIF-2 preparations is apparently inhibited by phosphorylation of the initiation factor (Fig. 2). As with the exchange reaction, the effect is totally dependent on the presence of both HCR and

ATP and is therefore unlikely to be an artefact caused by any GDP-binding or degradative activity of the HCR preparation used. There is evidence that the α subunit of eIF-2 is responsible for GTP and GDP binding¹⁸, so it is possible that incorporation of a phosphate group on this polypeptide interferes with both the entry and exit of GDP at the nucleotide binding site. Both processes could involve the putative guanine nucleotide exchange protein. Alternatively, the possible presence of unlabelled GDP already bound to the eIF-2 could prevent the association of ^3H -GDP with the factor, due to inhibition of

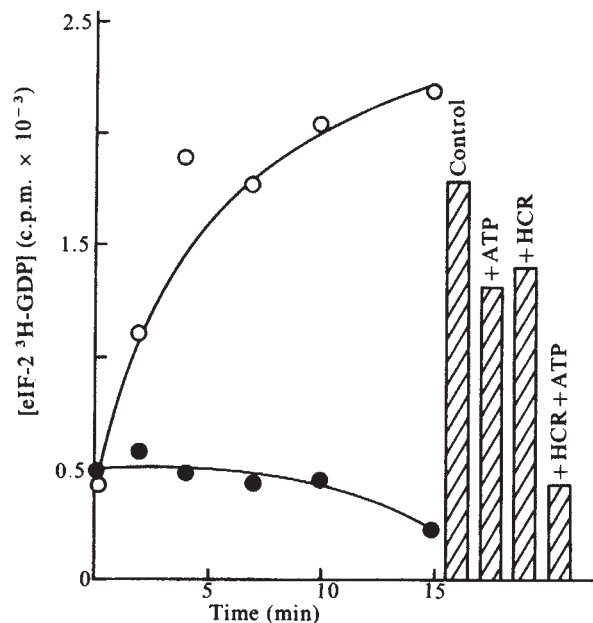


Fig. 2 Inhibition of GDP binding to eIF-2 by HCR-catalysed phosphorylation. The kinetics of binding of ^3H -GDP to eIF-2 were measured using the conditions described in Fig. 1 legend. Half the amount of eIF-2 was used and the initiation factor was incubated with 8 U ml^{-1} HCR and 0.2 mM ATP, where indicated, for 5 min before addition of the ^3H -GDP at time zero. Samples (20 μl) were withdrawn at times shown for assessment of ^3H -GDP binding as described in Fig. 1 legend. $\circ$, Control eIF-2; $\bullet$, eIF-2 treated with HCR and ATP. The histogram shows the requirement for both HCR and ATP in the inhibition of GDP binding, measured after 7 min of incubation.

Table 2 Requirement for ATP hydrolysis for inhibition of nucleotide exchange by HCR

Additions	³ H-GDP bound at:		% ³ H-GDP remaining at 30 min
	15 min	30 min	
	(c.p.m. × 10 ⁻³)		
ATP	7.45	1.17	16
ATP+HCR	8.25	3.42	41
Glucose + hexokinase	8.48	1.35	16
Glucose + hexokinase + HCR	8.65	1.08	12
AMPPcP	8.06	1.04	13
AMPPcP + HCR	9.02	1.85	21

Incubations were carried out as described in Fig. 1 legend, except that eIF-2 was incubated for 15 min at 30 °C with ³H-GDP in the presence or absence of 0.2 mM ATP or its non-hydrolysable analogue, AMPPcP, or of 10 mM glucose + 10 U ml⁻¹ hexokinase (to remove any traces of ATP present in other components). HCR was then added where indicated for a further 5 min, at which time the bound ³H-GDP was displaced by addition of 50 μM GTP + 12.3 nM unlabelled Met-tRNA_i. The data show the ³H-GDP bound to eIF-2 in 30-μl samples removed at 15 min (immediately before HCR addition) and 30 min (10 min after GDP and Met-tRNA_i had been added). Virtually identical data were obtained when ATP or glucose + hexokinase were added at 15 min rather than at zero time (not shown).

exchange after phosphorylation. The relatively slow kinetics of ³H-GDP binding to non-phosphorylated eIF-2 are consistent with such a possibility, although it is not known whether [eIF-2·GDP] complexes would remain stable through the several stages of purification involved in the preparation of the initiation factor.

In conclusion, we propose that phosphorylation of initiation factor eIF-2 inhibits the rate of initiation of protein synthesis by slowing the kinetics of exchange of GTP for GDP generated during 80S initiation complex formation. Although the results described here all concern the effects of phosphorylation catalysed by the haem-regulated protein kinase HCR, because the same site on eIF-2α is phosphorylated by the reticulocyte double-stranded RNA-activated protein kinase, and by a similar enzyme induced in numerous eukaryotic cell types by interferons, it seems probable that protein synthesis may be similarly regulated at the level of initiation in these systems.

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Maternally transmitted factors modify development and malignancy of teratomas in mice

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Maternal effects on the genome of the developing embryo have been well documented for amphibians^{1,2}. The existence of a similar maternal effect transmitted by the cytoplasm of the mammalian ovum has also been postulated³ but the evidence for this remains controversial and only one system, which demonstrates these maternal effects on development in the mouse, has been reported^{4,5}. Mouse embryos (7-day-old) transplanted under the kidney capsule of adult isogenic recipients give rise to either benign or malignant teratocarcinomas⁶, the yield of malignant tumours being high in some inbred strains but low in others⁷. In teratocarcinoma(TC)-permissive strains, such as C3H/He (C3H), or BALB/c, 50–70% of all embryo-derived tumours are malignant⁷, compared with only 10% in TC non-permissive strains, such as C57BL/6. This genetic non-permissiveness is not absolute and we have shown that the outcome of embryonic transplantations depends on both the embryo and the adult recipient of the graft⁶. We now present evidence that in reciprocal F₁ hybrids, TC permissiveness is determined by maternally transmitted factors. As these matroclinal differences were evident during early stages of embryonic development when the embryo had only briefly been in contact with the uterus, we believe that the differences are due to cytoplasmic factors transmitted in the ovum rather than metabolic maternal uterine effects.

We compared the weight of tumours and the ratio of teratomas to teratocarcinomas obtained from 7-day-old mouse egg cylinders of different derivation, transplanted into isogenic or F₁ adult recipients. The experiments were performed using embryos of two TC-permissive strains (C3H and BALB/c), one TC non-permissive strain (C57BL/6) and F₁ embryos obtained by reciprocal mating of TC-permissive and non-permissive parents. The embryos were transplanted under the kidney capsule of histocompatible female adult recipients and the tumours obtained from the embryos were collected after 2 months, weighed and histologically classified as either benign teratoma or malignant teratocarcinoma.

In the first set of experiments, we transplanted inbred embryos to either isogenic or histocompatible F₁ recipients (Table 1). The F₁ hybrids were produced by reciprocal matings to obtain recipient animals whose mother or father belonged to either a TC-permissive or non-permissive strain. The tumours obtained from transplants to isogenic and F₁ recipient animals were histologically similar but the ratio of benign to malignant tumours varied among different experimental groups. Table 1 shows that the permissiveness of C3H and BALB/c embryos was not altered on transplantation into F₁ hybrids and the ratio of teratomas to teratocarcinomas obtained from these embryos in F₁ hybrid recipients was essentially the same as in isogenic transplantations. In contrast, the weight of tumours obtained from both C3H (except group 4) and BALB/c embryos transplanted to F₁ hybrids was greater than that obtained in isogenic

Table 1 Percentage of malignant tumours obtained from 7-day-old inbred embryos transplanted under the kidney capsule of isogenic and F₁ hybrid adult recipients

Group	Embryo	Recipient	No. of grafts	Mean tumour weight (mg; $\pm$ s.e.m.)	Teratoma/teratocarcinoma	% Of malignant tumours
1	C3H	C3H	29	3,822 $\pm$ 460	15/14	48
2	C57BL/6	C57BL/6	30	1,319 $\pm$ 308	27/3	10
3	BALB/c	BALB/c	25	2,312 $\pm$ 210	9/16	64
4	C3H	(C57BL/6 $\times$ C3H)F ₁	37	3,410 $\pm$ 695	18/20	54
5	C3H	(C3H $\times$ C57BL/6)F ₁	40	5,436 $\pm$ 810	20/20	50
6	C57BL/6	(C57BL/6 $\times$ C3H)F ₁	49	2,714 $\pm$ 540	39/10	20
7	C57BL/6	(C3H $\times$ C57BL/6)F ₁	31	4,212 $\pm$ 781	15/16	48
8	BALB/c	(BALB/c $\times$ C57BL/6)F ₁	29	5,666 $\pm$ 682	6/23	80
9	BALB/c	(C57BL/6 $\times$ BALB/c)F ₁	27	3,780 $\pm$ 470	3/24	89
10	C57BL/6	(C57BL/6 $\times$ BALB/c)F ₁	28	1,740 $\pm$ 380	24/4	14
11	C57BL/6	(BALB/c $\times$ C57BL/6)F ₁	34	680 $\pm$ 240	30/4	12

Inbred mice C3H/He (C3H), C57BL/6 and BALB/c, and their F₁ female hybrids were obtained from Jackson Laboratories or produced in our animal colony. In F₁ hybrid mice, the female parental strain is shown first. The embryos were obtained from dated pregnancies. An embryonic portion of 7-day-old embryos was isolated and transplanted under the kidney capsule of adult isogenic or F₁ recipients. Recipient animals were killed 2 months after grafting and the tumours were weighed. Diagnosis of teratoma or teratocarcinoma was made histologically. Probability values for weight of tumours as calculated by Student's *t*-test were $P < 0.005$ (group 1 compared with 2, 2/7, 2/3 and 3/8); $P < 0.02$ (10 compared with 11); $P < 0.05$ (8/9, 1/5, 4/5). Probability values for the ratio of teratoma to teratocarcinoma, as calculated by χ^2 test were $P < 0.005$ (2 compared with 7, 7/10, 7/11); $P < 0.01$ (6/7); $P < 0.05$ (10/11); $P < 0.2$ (2/6).

Table 2 Percentage of malignant tumours obtained from 7-day-old F₁ hybrid embryos transplanted under the kidney of histocompatible F₁ hybrid adult recipients

Group	Embryo (F ₁ hybrid)	Recipient (F ₁ hybrid)	No. of grafts	Mean tumour weight (mg; $\pm$ s.e.m.)	Teratoma/teratocarcinoma	% Of malignant tumours
1	C57BL/6 $\times$ C3H	C57BL/6 $\times$ C3H	35	1,469 $\pm$ 433	29/16	17
2	C3H $\times$ C57BL/6	C57BL/6 $\times$ C3H	38	4,072 $\pm$ 850	20/18	47
3	BALB/c $\times$ C57BL/6	BALB/c $\times$ C57BL/6	29	5,686 $\pm$ 1,179	14/15	52
4	C57BL/6 $\times$ BALB/c	BALB/c $\times$ C57BL/6	24	1,460 $\pm$ 776	22/2	8

In F₁ hybrid mice, the maternal strain is shown first. Probability values for weight of tumours as calculated by Student's *t*-test were: $P < 0.005$ (1 compared with 2, 3 with 4). Probability values for the ratio of teratoma to teratocarcinoma as calculated by χ^2 test were: $P < 0.001$ (3 compared with 4); $P < 0.01$ (1 compared with 2).

transplantations. The non-permissiveness of C57BL/6 embryos as evidenced in isogenic transplantations (group 2) was abrogated in transplantation to some F₁ hybrids (group 7) but not to others (groups 10 and 11). Note that the ratio of teratomas to teratocarcinomas obtained from C57BL/6 embryos increased significantly on transplantation to (C3H $\times$ C57BL/6)F₁ hybrids whose mother was TC permissive (C3H) but did not increase in reciprocal (C57BL/6 $\times$ C3H)F₁ hybrids whose mother was TC non-permissive (C57BL/6). Paradoxically, the weight of tumours obtained from C57BL/6 embryos was lower in (BALB/c $\times$ C57BL/6)F₁ hybrids than in either isogenic or (C57BL/6 $\times$ BALB/c)F₁ recipients, although the ratio of malignant to benign tumours did not change. These data indicate that the TC non-permissiveness is relative and may be modified by the host. It is also obvious that in reciprocal hybrids the malignancy of embryo-derived tumours is determined by the parental derivation of the recipients. The increased yield of malignant tumours and accelerated growth of tumours occurred only in the F₁ hybrids whose maternal strain was TC permissive (C3H).

In the second set of experiments, we compared weight of tumours and the ratio of malignant to benign teratomas obtained after transplantation of F₁ hybrid embryos to histocompatible F₁ recipients derived from matings of TC-non-permissive C57BL/6 strain and the TC-permissive strains (C3H and BALB/c). In each combination, the materno-paternal strain derivation of recipient animals was kept constant and the derivation of embryos was reciprocal. Table 2 shows that the embryos whose maternal strain was TC non-permissive (C57BL/6) gave rise to teratocarcinomas in essentially the same ratio as the inbred C57BL/6 embryos. In contrast, the embryos whose mothers were TC permissive and the fathers TC non-permissive ((C3H $\times$ C57BL/6)F₁ and (BALB/c $\times$ C57BL/6)F₁ embryos) gave rise to significantly more teratocarcinomas than the reciprocal hybrid embryos. The ratio of teratoma to teratocarcinoma in the two latter groups corresponded to the ratio expected from isogenic embryo transplants of C3H and BALB/c (maternal strain) embryos.

The F₁ adult recipients and embryos produced by reciprocal mating of a TC-non-permissive and two TC-permissive strains have identical genomes notwithstanding the Y chromosome

present in ~50% of the embryos. Nevertheless, the outcome of embryonic transplantations was markedly influenced by the maternal strain of the F₁ hybrid recipient animals and even more so by the derivation of F₁ hybrid embryos. These differences indicate a matroclinal effect, but additional studies are needed to determine whether this effect is due to the maternal environment during embryonic development (the 'uterine effect') or 'cytoplasmic factor(s)' transmitted in the cytoplasm of the ovum³. The present data do not rule out either of these hypotheses. The experiments with F₁ hybrid embryos indicate that the differences between reciprocal hybrids are already evident in the early postimplantation stages of embryonic development, thus favouring the existence of cytoplasmic factors, as it is unlikely that external, maternal metabolic influences could have modified so profoundly the developing embryo, as it had only briefly been in contact with the maternal organism before transplantation.

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Errata

In the letter 'T-cell mitogens cause early changes in cytoplasmic free Ca²⁺ and membrane potential in lymphocytes' by R. Y. Tsien, T. Pozzan and T. J. Rink, *Nature* **295**, 68–71 (1982), on page 69 the concentration of the phosphodiesterase inhibitor Ro20/1724, should be 10 μ M and that of dibutylryl cyclic AMP should be 0.25 mM.

In the letter 'Atmospheric angular momentum and the length of day: a common fluctuation with a period near 50 days' by R. B. Langley *et al.*, *Nature* **294**, 730–732 (1981), on page 732 the acknowledgement should read that R.B.L. was supported in part by a Natural Sciences and Engineering Research Council of Canada Postdoctoral Fellowship.

BOOK REVIEWS

An inspection of inspectorates

Eric Ashby

A BOOK written under the surveillance of a committee, setting out the results of a project supported by a grant from the Social Science Research Council: surely the kiss of rejection for book-reviewers! Perhaps that is why this book has received so little attention. I am glad to have the chance to redeem this neglect. For the book does not have that bland anonymity associated with such multiple parentage. The committee was a working group set up by the Royal Institute of Public Administration to assist Mr Rhodes in his research; but they left Mr Rhodes on his own to write the book and it has the imprint of a man of independent mind examining the material he has collected and coming to clear and well-argued conclusions about it. The conclusions deserve careful study and further discussion.

At the end of his book Mr Rhodes asks (though not as bluntly as this) "Are inspectors really necessary?". He explains how difficult it is to get an answer to the antecedent question: "What good do they do?", and he shows how one needs to dig into their history in order to appreciate their present usefulness. There are two kinds of inspectorates; those that have to ensure compliance with statutory requirements (enforcement inspection) and those that have to maintain or improve standards of performance (efficiency inspection). At extreme ends of the scale of inspectorates there are clear differences between these two categories: an inspector of weights and measures has to enforce the law that grams and litres used in trade are to be uniform all over the country; an inspector of schools does not have to enforce compliance with any statutory standard. But — and this is what makes the topic of inspectorates so complex and so controversial — history demonstrates that the most effective enforcement inspectorates do not depend for their efficacy on enforcement (in the literal sense). They do have the power to bring culprits to court, and on paper it may seem that their job is to act like policemen enforcing a 30-mile-an-hour speed limit. But that is not the way they work. Even factory and mines inspectors, up against bitter opposition from mill and mine owners who resented interference from the state, learnt early in their history that they got the best result from being (as the chief inspector of factories in 1878 put it) "the friend of the manufacturer as much as the friend of the *employé*". The first alkali inspector, charged in 1863 to enforce a 95 per cent reduction in the emission of hydro-

Inspectorates in British Government: Law Enforcement and Standards of Efficiency. By Gerald Rhodes. Pp. 281. ISBN 0-04-351056-6. (George Allen & Unwin: 1981.) £15, \$35.

chloric acid from soda works, came to the same conclusion: I work, he wrote, "at present, by advice and friendly admonition, and prosecutions will come in their proper time".

Except for flagrant and defiant offenders, the time for prosecutions never has come. This is in striking contrast to the style of administration in the United States. In the control of pollution, for example, there are at any one time hundreds of cases in the courts in the USA, compared with five or ten at most in Britain. What is the reason for this difference? One reason is that inspectorates in Britain have acquired, in over a century of practice, a discretion to make what are really quasi-political decisions, using their judgement in the way they interpret their powers under the law. There is no provision in law (for instance) for an obsolete cement works, soon to be closing down, to be allowed a greater licence to pollute than a new cement works, just being put into operation. If the alkali inspector had no more discretion than a policeman, he could not allow such licence. But — as Mr Rhodes cogently argues in his book — it is just because the alkali inspector (and the same holds for other sorts of enforcement inspectorates) has been allowed discretion that his work is, on the whole, so effective in the public interest. Inspectors who use their discretion in such ways as this are, of course, vulnerable to the criticism that they are in the pocket of the manufacturer. But on balance this tradition in British inspectorates — a discretion to compromise and bargain cooperatively with the persons being inspected — has stood the test of a century of practice.

"Are inspectors really necessary?". Of course Mr Rhodes does not answer this question, but he leaves the reader with a pretty persuasive hint that he would say: "For the present, yes". Inspectors for efficiency, in particular, help to keep standards up to the mark; and even though there is no reliable way to measure their value quantitatively, the fact that they are highly valued by the very people whom they inspect is sufficient to justify their maintenance. Everyone who is liable to be inspected (and I guess that covers most

readers of *Nature*) should find time to read this admirable book. It explains how a race of officials who in some countries are the object of denigration, have — thanks largely to the perception and tact of their Victorian predecessors — become in Britain the object of respect. □

Lord Ashby was formerly Master of Clare College, Cambridge. His most recent book, written with Mary Anderson, is The Politics of Clean Air (Clarendon/Oxford University Press, 1981.)

Sense of déjà vu

H.F. Rowell

Sense Organs. Edited by M.S. Laverack and D.J. Cosens. Pp. 394. ISBN 0-216-91094-3. (Blackie: 1981.) £29.75.

THE editors of *Sense Organs* state their object to be to produce

a set of stimulating contributions covering what is known about how biological sensors work, their sensitivities, their ambiguities, their input to the CNS and the manner in which their signals are interpreted.

Their attempt at this enormous task amounts to less than 400 pages, and not surprisingly the result is gappy and uneven. The book consists of papers presented to the Scottish Electrophysiological Society in 1980. The contributors appear to have been drawn from those in the field who were available in Britain or Western Europe at the time, and one suspects that the immense scope aimed for by the editors was determined by the diversity of the contributors, rather than the reverse.

The patchiness of the contents is accentuated by irrational ordering. For example, an article on behavioural correlates of photoreception is placed under "Structure and Function of Sense Organs", and within each of the book's two subsections there is no grouping of articles by either modality, phylum or level of analysis. Several contributions are traditional in style: the Introduction and the Epilogue by Autrum and Loewenstein, respectively, an article on sensory coding by Bullock, one on cognition by Gregory, one on "unusual" (i.e. speculative) senses by Brown, and a general essay on modalities by one of the editors. These or their near equivalents have appeared at most

symposia in this area for the past 20 years. The remainder are of two types: general reviews of a sub-field (thus Boekh on chemoreceptors, Sandeman on equilibrium systems in arthropods, or Hawkins and Korner on the acoustico-lateralis system, to mention three of the best), or descriptions of the author's own work. Some articles which at first appear to be reviews are actually of the second type. Of the accounts of individual work, some of the best have already, and deservedly, had extensive public exposure (for example Land on a phylogenetic dichotomy in the optics of Crustacea, and Kirschfeld on the role of photostable pigments in vision) and produce a sense of *déjà vu* when encountered again with the long latency of a symposium volume.

However, I found three articles stimulating. Young gives a fascinating account of his research on the bizarre eye of *Daphnia*, and produces biologically satisfying explanations for many of its peculiarities. Light from above appears to be recognized by its high proportion of blue, side light in alga-rich water by green colour: appropriately sensitive and spatially organized receptors coupled with suitable behaviour enable the animal to stay the right way up and near to the surface, and to find and exploit algal patches. The author also convinces one that the "tremor" characteristic of this and similar eyes is unrelated to the interommatidial angle and hence not directly subserving scanning. Stieve reviews the role of calcium in visual transduction in invertebrates, and den Otter transduction in chemoreceptors. Both accounts are lucid and interesting and include much that is unfamiliar to me. As a neuron — rather than a receptor — physiologist I found the variety of off-beat mechanisms considered striking. Thus the control of light-activated channels in photoreceptors is attributed to a Ca/Na antagonism with competition for identical binding sites, and the possibility is entertained that the receptor potential of chemoreceptors may communicate with the spike initiating zone not electrotonically but via conformational change of microtubules or even via compression waves in the plane of the cell membrane.

I have slowly come to the view that the published symposium is the least useful form of scientific publication, except possibly for the isolated graduate student in a forgotten university. In general it consists only of material more adequately treated either in research articles or in review volumes. Most of *Sense Organs* reinforces this opinion — the useful bibliographies and the few articles which, as the editors put it, genuinely "convey the fascination and excitement of the topic" seem barely worth the tedium of the remainder. □

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Astronomy: the politics and engineering

C.M. Humphries

Telescopes for the 1980s. Edited by G. Burbidge and A. Hewitt. Pp.278. ISBN 0-8243-2902-3. (Annual Reviews, Palo Alto, California: 1981.) \$27 (US), \$28 (elsewhere).

NOT least among the ingredients required to bring a major national project from concept to fruition is perseverance. Budget trimming due to inflation, committee wrangles, procrastination and politics are just some of the adversities which may have to be weathered and which are all too familiar it seems, judging by the accounts of four large astronomy projects recently undertaken in the United States. Two of the projects described in *Telescopes for the 1980s* are ground-based telescopes and two are space observatories; together, they span the spectral region from radio frequencies to X-rays.

The Very Large Array was conceived in the early 1960s when it was recognized that existing radiotelescopes in the USA were incapable of extending extragalactic studies much further. Spurred by the need to create an imaging instrument with improved sensitivity and resolution, the last of the 28 movable 25m dishes (27 + 1 spare) was completed 18 years after the initial design studies began. In his account of the project, D.S. Heesch comments that "in spite of real problems, petty irritations, miscellaneous distractions and excessive inflation, VLA construction proceeded pretty much on schedule and within budget". The result is a powerful aperture synthesis telescope with 351 separate baselines and an angular resolution of 0.1–2 arcsec depending on the wavelength band used.

The Multiple Mirror Telescope, described by authors from the participating groups at Tucson and the Smithsonian

Astrophysical Observatory, is the subject of the next section. By comparison with the others, the MMT programme seems to have benefited by having a fairly loose organizational structure in its early years. Personal recollections by R.J. Weymann, former Director of Steward Observatory, give a nice insight into how that collaborative structure evolved and why it worked. On the matter of funding, the project was severely criticized at one stage for having started before financial backing was fully identified. Weymann's response to this is that "given the particular set of circumstances surrounding the MMT, to wait until funding had been located would have been tantamount to abandoning the project". A highly innovative optical and IR telescope was the result, and one which has an exciting new potential for speckle interferometry with the individual mirrors co-phased.

C.R. O'Dell's description of the Space Telescope project gives a fascinating account of the way in which new programmes evolve within NASA and of the relationship between NASA, the Office of Management and Budget (part of the executive branch of government) and the House of Representatives appropriation subcommittee. At one stage the ST was deleted entirely from the approved NASA budget for the financial year 1975 ("since the costs exceeded the choke limit of Congress"). When the project was later reinstated, the budget was less than NASA had requested, forcing a reduction in aperture to 2.4m and a re-examination of the scientific performance goals. Despite the compromises which had to be made, the 0.1 arcsec imaging capability still promises a huge observational advance.

The Einstein space observatory,



The Very Large Array — 27 mobile dishes, at maximum extension providing the equivalent of a radiotelescope 21 miles in diameter.

discussed in the final section by R. Giacconi *et al.*, was launched in November 1978 and completed its operational lifetime 2½ years later. It, too, experienced some potentially disastrous setbacks (and had to be reduced in aperture by a factor of two) yet survived to make important X-ray observations with unprecedented sensitivity and an angular resolution (1–2 arcsec) which now matches that attainable with ground-based optical telescopes and the VLA.

Each of the sections in *Telescopes for the 1980s* contains a detailed account of the design and performance of the telescope and its instrumentation, and the sections on the VLA, MMT and Einstein observatory present examples of the scientific results obtained up to the end of 1980 (descriptions of the scientific uses of the ST are available elsewhere). The text is well supplied with diagrams and tabulated material, making a volume which is both useful and informative. □

Colin Humphries is a Principal Scientific Officer at the Royal Observatory, Edinburgh, and was Project Manager for the construction of the United Kingdom Infrared Telescope.

Cinderella subject

P.G. Williamson

Palaeoecology, Concepts and Applications. By J.R. Dodd and R.J. Stanton. Pp.544. ISBN 0-471-04171-8. (Wiley: 1981.) £29.55, \$53.15.

THE renaissance that has swept large areas of palaeontology over the past two decades has left palaeoecology curiously untouched. New concepts in evolutionary palaeontology, such as punctuated equilibrium theory, are currently subjects of lively controversy within general evolutionary biology: palaeontologically-based approaches to morphological analysis — such as Seilacher's 'Konstruktionsmorphologie' — are major influences on neontological interpretations of functional morphology. Palaeobiogeographical investigations have provided significant insights into the development and relationships of modern faunas. Increasingly, these various fields of palaeontological investigation have made significant contributions to their neontological sister-disciplines. Up to now, palaeoecology has been a sad Cinderella, absent from this intellectual ball. The main interest of this book is that it indicates — in part at least — why this is so.

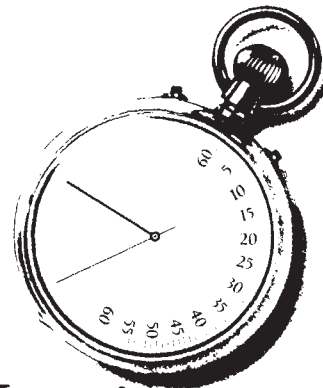
The authors' avowed intent is to illustrate the utility of palaeoecology in palaeoenvironmental reconstruction. To this end, their book consists largely of a long series of anecdotal summaries of

previously published palaeoecological analyses. Chunks of basic ecological theory are periodically interjected; these are followed by further speculations as to how insights from modern ecology might, with varying degrees of ambivalence, assist palaeoecological reconstruction in particular instances. This approach has the unfortunate effect of revealing palaeoecology as a largely derivative field, a poor-man's applied ecology performed on inadequate data.

The dreary state of the discipline is best illustrated by those cases in which intensive work over several years has permitted the comprehensive characterization of given palaeoenvironments. A case in point is Stanton and Dodd's own exhaustive — and entirely admirable — work on the Pliocene Kettleman Hills deposits of the San Francisco area. This work is used as a connecting thread to link the various palaeoecological techniques explored in successive chapters (a good idea, incidentally). Assiduous application of various techniques over several years has allowed a detailed understanding of conditions in the Pliocene marine embayment of the Kettleman Hills. The upshot of all this effort seems to be that palaeoenvironments in the Kettleman Hills during the Pliocene were, well, rather as you might expect. Salinities varied from time to time and area to area. The biota varied correspondingly and predictably from time to time and area to area. This kind of empirical analysis could be repeated a million times in the hundreds of millions of cubic kilometres of the global Phanerozoic rock record, but hardly seems a potentially fruitful research strategy for a science aspiring to an original conceptual framework.

Admittedly, these are problems of the discipline as much as of the book. But the book itself suffers from serious shortcomings. These are sins of omission rather than commission, but are problems nonetheless. There is virtually no discussion of the plethora of statistical techniques that have so usefully been applied to specific palaeoecological problems in recent years. This is particularly unfortunate as the book claims to emphasize the applications of palaeoecology. To this extent — as a teaching aid — the book compares unfavourably with such texts as Reymont's *Introduction to Quantitative Palaeoecology* (Elsevier, 1971). Most discussion of numerical techniques is superficial: this supposedly graduate-level text provides no usable explanation of rarefaction! Discussion of techniques for the measurement of faunal diversity — surely no small matter in palaeoecological studies — merits a scant four pages, and Fisher's parameter α isn't even mentioned. You do not have to be a rabid Hennigian to wonder why an entire chapter, supposedly devoted to palaeobiogeography, lacks any reference to vicariance biogeography.

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Stanton and Dodd provide a useful general survey of the palaeoecological literature. But this survey is rendered somewhat inaccessible by the fact that there is no author index. The book is overlong for what it has to say, and there is an unfortunate tendency towards Haig-speak: "Using the community . . . as an information transfer unit is most effective in the Neogene"! The book could be two-thirds of its length and still be an equivalent

"information transfer unit".

I suggested previously that palaeoecology has been the absent Cinderella at the modern palaeontological ball. Stanton and Dodd's book suggests that she may have some time to wait for the Good Fairy, never mind the glass slipper. □

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Spot the potential carcinogen

Alastair Hay

Evaluation of Short-Term Tests for Carcinogens. Progress in Mutation Research, Vol.1. Edited by Frederick J. de Serres and John Ashby. Pp.827. ISBN 0-444-00570-6. (Elsevier/North-Holland: 1981.) Dfl.295, \$125. *Short-Term Tests for Chemical Carcinogens.* Edited by H.F. Stich and R.H.C. San. Pp.518. ISBN 3-540-90496-4. (Springer-Verlag: 1981.) DM 142, \$64.60.

CANCER caused by contact with chemicals at the workplace and in the environment is something regulatory authorities are trying to control. The task is a daunting one. New chemicals reaching the market place each year are numbered in the thousands. Yet each chemical must be tested to assess its toxicity, and in particular its potential for causing cancer. Can this be done using tests that will satisfy the regulators, but will not bankrupt industry? And, just as important, are there assays which will give an answer in days, as opposed to the two years for the standard animal carcinogenicity study?

There would seem to be an optimistic answer to both questions. There are tests which satisfy these criteria and which can be used to predict carcinogenic activity. The first of the so called "short-term tests" for carcinogens was originally developed by Dr Bruce Ames at the University of California (the Ames test). Using bacterial strains of *Salmonella*, Ames showed that chemicals which caused cancer in animals would cause bacteria to mutate. This ability to cause mutation, he argued, could be exploited to test for carcinogens. Thus a chemical which was mutagenic in the *Salmonella* test would be considered to be a potential carcinogen.

Unfortunately it is not that simple. Neither the Ames test nor the others developed since its discovery — based on the mutation of other bacterial, fungal or mammalian cells — will identify every potential carcinogen. The short-term tests are fickle. They give better results for one class of chemical than for another. In addition, many of the tests have been evaluated with the researcher knowing both the identity of the chemical being

tested and that it is carcinogenic in animals.

This kind of information is not going to be available when new chemicals are being tested. In this case, the tests will have to predict which chemicals are likely to cause cancer. Until recently short-term tests had never been evaluated under conditions in which they would be used by regulatory authorities to identify problem chemicals.

In 1976, however, a start was made to remedy this situation. *Evaluation of Short-Term Tests for Carcinogens* is the result of that initiative. It is the report of a unique international programme of collaboration between scientists in many countries to look at the ability of these tests to predict carcinogenicity. The venture, started by the UK Medical Research Council and Health and Safety Executive, was broadened to include scientists from ICI's Central Toxicology Laboratory and the US National Institute of Environmental Health Sciences. The US Environmental Protection Agency and the National Cancer Research Institute in Japan also made a financial contribution to the study.

Scientists in some 60 laboratories were sent 42 chemicals, 25 carcinogens and 17 non-carcinogens. The chemicals were coded and the identities were only revealed after the investigators had submitted their final results. According to the organizers, the chemicals were selected on the basis of the information available about their carcinogenicity in animals. Examples from most of the major classes of organic chemical carcinogens were chosen, as were chemicals which are either "vital to life or are considered essential to our modern lifestyle". This latter group included three chemicals assumed to be non-carcinogens such as methionine, vitamin C and household sugar.

Because of the design of the study, the 30 or so tests under scrutiny were being evaluated as predictors of carcinogenic activity and not just as tests to look at the mutagenic properties of chemicals. Thankfully, the results of this expensive but unique exercise in collaboration show that the tests have an important role to play in any programme to screen for potential carcinogens.

In the introductory chapters to this book the editors set out the reasons for the study; the basis for selection of chemicals used; the animal carcinogenicity data on these chemicals; and a summary of the results obtained by the laboratories. In the remaining 60 or so chapters each investigating team describes their own experimental protocol and discusses the results obtained in detail.

The book contains a wealth of material which will be of immense value to anyone interested in identifying chemicals which are potential carcinogens. The results of the study show that the most effective tests are those which use bacteria such as *Salmonella* or *Escherichia coli*. However, these bacterial tests do fail to pick out some potential carcinogens which other fungal or mammalian tests will identify. But these other tests in turn fail to identify chemicals which the bacteria pick out. All tests have false negatives and false positives and the study shows that a battery of tests will be required for satisfactory results.

The unequivocal evidence about a chemical's potential for causing cancer is always regarded as being provided by tests in animals; indeed this study was based on that premise. It is somewhat ironic, therefore, that the study has shown that animal carcinogenicity data are not always as good as they might be. Azoxybenzene is said to be a non-carcinogen in animals yet many of the *in vitro* tests identify it as a potential carcinogen. According to one of the editors, John Ashby, this suggests that the negative carcinogenicity data on this chemical which are available may not be reliable. It is recommended that azoxybenzene should be re-tested in animals.

Imaginative titles for books on screening tests for carcinogens are obviously in short supply. Neither of the two books reviewed here has what one might call an arresting label. But the titles do at least tell the reader what he or she is going to find. The second book, *Short-Term Tests for Chemical Carcinogens*, contains papers from 43 experts in the field, some of whom participated in the international study. The papers describe different tests in detail and the evaluation of some of them in individual laboratories.

The value of this second volume is in the scope it gives to authors to discuss the more neglected area of viral tests for carcinogens. And some authors address the complex issues of anticarcinogens, cocarcinogens, promoters, sensitizers and DNA repair inhibitors. The role these other factors play in the mechanism of carcinogenicity is still far from clear. However, with the research effort now being devoted to this area answers will not be long in coming. These two books show that the effort so far has been worthwhile, and that the short-term test has a vital part to play in detecting potential carcinogens. □

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